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Future feeds in aquaculture: insects as a new ingredient for fish culture

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Introduction

1.1

The aquaculture sector

Aquaculture is the rearing of aquatic organisms under controlled or semi-controlled conditions which involves animal and plant culture [**1**]. This sector represents the food production technology with the fastest expansion, with an average annual growth rate of 5.3% during the period 2001-2018 and which continues to dominate the aquatic feed and food production globally [**2**,**3**].

In 2018, global aquaculture production reached 82,1 million tons of which 62% were produced by inland facilities and 38% by coastal plants and mariculture [**2**]. The largest fraction of aquaculture production consisted of fish (47.7% of total aquaculture), the majority of which are represented by freshwater species [**4**]. Fish derived from capture fisheries and aquaculture represent the largest source of animal protein worldwide. Global food fish consumption has been shown to increase at an average annual rate of 3.1% from 1961 to 2017, doubling the annual world population growth (1.6%) of the same period and higher than all the other animal protein foods [**2**]. However, capture fisheries production has been stagnated since the late 1980s due to the overfishing and the inability to sustain the increasing global food fish demand [**2**,**5**]. In light of the static condition of fisheries sector, aquaculture can be considered the major responsible for the supply of fish for human consumption, providing about 3-2 billion people with 20% of their animal protein intake [**2**,**6**].

However, the global population is projected to reach 9.7 billion people by 2050 and aquaculture sector is challenged to reach the 109 million tons in 2030 and 140 million tons in 2050 [**5**,**7**]. Because of stagnation of capture fisheries, the further aquaculture expansion represents the only available solution to achieve these remarkable targets, but the growth of this important food-production sector may cause adverse environmental effects [**8-10**]. Aquaculture expansion has already been associated with negative ecological impacts like greenhouse gas emissions, widespread conversion of wetlands (mangroves, coastal lakes and lagoons) to inland fish farm and the not voluntary introduction of non-indigenous farmed fish species led to irreversible habitat alteration or destruction that negatively impacts local biodiversity [**5**,**11**,**12**]. The release of chemicals, waste waters, untreated nutrients and organic matter release into open-water environment can negatively affect aquatic ecosystems [**13**].

Chemical and medicine treatments are potential causes of water pollution, while the high densities and overfeeding in intensive aquaculture facilities can result in high amounts of organic matter and fish waste that led to a severe reduction of dissolved oxygen concentration through their decomposition processes [**5**]. Aquaculture might significantly contribute to nutrient pollution with

consequent eutrophication phenomena which cause adverse effects on both farmed fish stocks and wild fish population through harmful algal blooms [**13**].

These ecological issues have been faced with the development of environmental-friendly fish culturing systems and with the funding of several research project aimed to improve aquaculture sustainability in light of a circular economy concept. As an example, integrated multitrophic aquaculture combine fish, shellfish and detritivores organisms like holothurians (organic extractive species) and seaweeds (inorganic extractive species) culture to restrict the nutrient and organic matter dispersion [**14**,**15**]. Differently, to minimize the direct interaction between the production processes and the environment, recirculating aquaculture systems (RAS) represent an eco-friendly and increasingly proposed strategy [**16**]. RAS are land-based, indoor fish rearing facilities, in which tanks are located within a controlled environment and water is filtrated to be purify from metabolic wastes of stock, before being recirculated into the system itself [**16**]. Finally, aquaponics represents a small but rapid growing sector that promote quality food production combining fish culture and plants production, saving energy, water and nutrients to move towards a zero-waste generation [**17**].

Besides the reduction of environmental footprint through the development of more efficient fish culture systems, one of the ongoing challenges of aquaculture sector is the increase of aquafeed production sustainability. For a long time, fish meal (FM) and fish oil (FO) have represented the gold standards in aquafeed formulation, but the overexploitation of pelagic fisheries and increasing costs pose severe limitations in their use [**18**].

1.2

Fish meal and fish oil: the gold standards in aquafeeds formulation

FM and FO have been used in several types of feeds for farmed animals but have proven particularly valuable for aquafeed formulation [**19-21**]. These ingredients are generally manufactured from wild-caught, small marine fish which are not suitable for direct human consumption due to their high percentage of bones and oils. The main fish species rendered into FM and FO are anchovies, herrings, menhaden, sardines, shads and smelts [**22**]. These fish species are considered "industrial" since their capture is for the exclusive of the production of these ingredients, while a small percentage derived from by-catch or by-products originated by seafood processing [**23**]. FM represents the major source of protein in fish diets: high-quality FM contains 60 to 72% crude protein by weight [**24**,**25**]. The nutritive value of the protein relates directly to its amino acid composition and digestibility especially for monogastric animals that only have dietary requirements for specific amino acids rather than protein amounts [**25**,**26**]. FM is a precious source of the ten essential amino acids for fish (arginine, histidine, isoleucine, leucine, lysine, methionine, phenylalanine, threonine, tryptophan and valine), especially for the high sulphur-containing ones. FM also lacks of antinutritional factors if properly produced and stored, and has limited carbohydrate and fibre content [**19**,**27**]. Finally, FM is highly palatable to cultured fish species, a key-property to ensure rapid growth and reduce nutrient leaching due to rapid ingestion [**28**].

FO is extracted during the rendering of FM and represents an excellent source of polyunsaturated fatty acids (PUFA), particularly those in the n3 series, which are extremely important for fish [**29**,**30**]. PUFA are required for cellular membrane structure and function since they constitute phospholipids, the fundamental components of lipid bilayers. Particularly, docosahexaenoic acid (22:6n3; DHA) plays an important functional role in neural, visual and sperm cells of fish due to its structure [**31**,**32**]. In fact, with six methylene-interrupted double bonds, DHA provides a strong, but at the same time flexible, structure that can support the rapid and repeated membrane re-arranging of particular cell types like neurons [**32**]. Furthermore, PUFA are involved in the regulation of fish cellular metabolism and physiology. The regulated oxidation of both arachidonic (20:4n6; ARA) and eicosapentaenoic (20:5n3; EPA) acids led to the production of highly bioactive eicosanoids that possess a wide range of physiological actions in blood clotting, immune and inflammatory responses, cardiovascular tone,

renal and neural functions, and reproduction [**33**,**34**]. PUFA originate in aquatic food webs from primary producers. In particular, marine microalgae are able to synthesize and store high levels of highly unsaturated fatty acids, like EPA and DHA, while freshwater phytoplankton are characterized by high levels of linoleic (18:2n6) and α-linolenic (18:3n3) acids [**35**]. For that reason, dietary PUFA requirement differs between freshwater and marine fish species. Marine fish species have had no evolutionary pressure to retain the ability to endogenously produce highly unsaturated fatty acids (HUFA) and they must be provided through the diet. Conversely, freshwater fish PUFA requirement can be generally satisfied by linoleic (18:2n6) and α-linolenic (18:3n3) acids since they possess the biosynthetic pathways to further desaturate and elongate these C18 PUFA into more highly unsaturated ones (like EPA and DHA) [**32**].

Aquaculture still depends on on the proper supplementation of PUFA ensured by FM and FO for the rearing of both: (i) marine species that lack the HUFA biosynthetic pathway and (ii) freshwater species since *de novo* synthesis of any non-essential nutrient consumes energy that, in fish culture context, would be better used to support somatic growth or reproductive processes [**36**].

FM and FO were originally used because were considered inexpensive and palatable sources of protein and lipid, but in the last decades, the incorporation of wild-caught fish in aquafeeds triggered environmental and economic concerns [**18**]. The growing demand of FM and FO for aquafeeds formulation has been progressively identified as contributor to the overexploitation of natural fish stocks [**18**]. In addition, another key-factor that has limited the use of FM and FO has been the rising cost of these raw materials. In fact, their constant demand in an ever-growing aquaculture sector coupled with a relatively static supply of fisheries, has resulted in prices increase [**2**]. In light of this, there are considerable environmental and economic incentives to reduce the dependency on limited marine resources in order to allow aquaculture to produce healthy food in a sustainable way [**37**]. Scientific community has been moved to the research of alternative protein sources but, whereas rearing of herbivorous and omnivorous fish species has readily transitioned to diets containing ever lower FM and FO levels, such aquafeed formulations have been more difficult to apply in carnivorous fish culture [**38**]. In addition, the main bottleneck in the use of FM/FO-free formulations is represented by the HUFA supply of the alternative ingredients which is still not comparable to that of marine resources [**39**]. Long-term dietary PUFA and HUFA deficiency results in reduced growth and increased mortality in fish [**32**]. In addition, other pathologies linked to a dietary lack of PUFA include myocarditis, hepatic and intestinal steatosis, fin erosion, bleeding from gills, lordosis, reduced reproductive performances, behavioural impairment and shock syndrome have been demonstrated [**32**,**40**]. In addition, this deficiency may affect the nutritional composition of the edible portion. Fish

represent one of the main sources of PUFA content in human nutrition that are well known to reduce the risk of cardiovascular and inflammatory disorders [**41**].

A variety of marine-derived oils has proven to be potential FO replacements. Some are derived from wild-caught marine organisms like krill or by fisheries by-products (seafood processing wastes or by-catch), but the promise of these raw materials is constrained by the same factors that incentivize the reduce reliance on traditional FO. Furthermore, their inconstant availability suggest to think of these ingredients as feed supplements instead of FO replacers [**21**,**42**]. In the last decades, the main challenge of aquaculture sector has been the reduction of the reliance on marine resources for aquafeed production by identifying and testing cost-effective and more sustainable alternatives, but results have been controversial, depending on several factors like fish species tested, their nutritional requirements and life-cycle stage as well as the type and degree of inclusion respect to FM and FO of alternative ingredients tested.

1.3

The available alternatives over the last decades: advantages and drawbacks

Aquaculture sector needs to become sustainable and globally competitive through the advancement of knowledge on fish nutritional requirements and the continuous search of sustainable raw materials that can solve strategic problems in fish nutrition [**43**,**44**]. During the last decades, great efforts have been done to develop less expensive and more sustainable alternative protein and lipid sources respect to FM and FO, without compromise fish welfare and nutritional value [**39**].

Plant-derived ingredients

Incorporation of plant ingredients in aquafeed formulation is a widely used practice mainly driven by higher abundance and lower price compared to FM and FO [**39**]. In the last two decades, the plant protein-rich feeds like oilseed meals, cereal glutens, protein concentrates and pulses, became the major protein sources in fish diets, mostly substituting FM [**19**]. However, even if plant protein-based diets highlighted promising results in different fish species, they are often in direct competition with human nutrition and have been shown to affect fish growth and welfare [**45**,**46**].

The amino acid profile of plant-derived ingredients does not completely match the essential amino acid requirement of fish, and this often makes necessary an amino acids supplementation to compensate the deficiencies [**47**,**48**] or a combination of plant-based ingredient to achieve the correct fish amino acid balance [**43**]. One of the major solutions is represented by the mixture of corn gluten (high in methionine but low in arginine and lysine) and soybean (high in arginine and lysine, but low in methionine) meals, but these kinds of combinations can trigger some adverse side-effects due to the presence of another unfavourable component of plant-derived ingredients, the anti-nutritional factors (ANF) [**20**,**39**]. ANF are substances that can interfere with nutrient digestion and absorption, reduce feed utilization and palatability, and severely affect fish gut health [**19**,**26**,**49**]. Adverse effects due to ANF dietary presence with consequent impairment of growth performances have been widely described in salmonids in which several studies have shown that high inclusion levels of soybean meal in the diet often caused non-infectious enteritis onset in the distal intestine [**50-53**] characterized by lymphocytes and granulocytes infiltration, shortening and reduction of mucosal folds, decrease of enterocytes supranuclear vacuoles and thickening of the lamina propria [**54-57**]. It should be pointed

out that the enteritis-responsible factors can be removed or deactivated during the processing that extract carbohydrates from soybean meal to obtain purified soy protein concentrates [**20**,**58**]. Differently, some ANF like phytic acid, phenols and tannins remain intact after processing methods and their effect should be mitigated by feed supplements [**39**]. Phytic acid represents the storage form of phosphorous in seeds. Phosphorous in this molecule is not available for monogastric animals and passes through the gastro-intestinal tract (like non-soluble carbohydrates and fibres from plant-derived ingredients) enriching ponds or water basins in which farm effluents are discharged, contributing to eutrophication [**20**,**59**]. Furthermore, phytic acid ties up divalent cations under certain conditions, making them unavailable to fish that can become deficient in essential minerals, especially zinc [**20**]. The reduction in the feeding and growth with response to higher levels of dietary plant proteins has been reported in several fish species like rainbow trout [**60**,**61**], European sea bass [**62**,**63**], Atlantic salmon [**64**], and gilthead sea bream [**65**]. The main causes were related to the less apparent digestibility coefficient, intestinal damage, essential amino acid deficiency, less palatability, presence of ANF, and elevated muscle protein degradation [**19**,**66-68**]. In light of the obtained results, the inclusion of plant protein sources in the diet respect to FM able to guarantee a proper growth and feed intake varies within fish species [**69-73**].

Considering vegetable oils, the steadily increasing production, high availability and stable prices are features that currently make them the most sustainable alternatives for FO replacement in aquafeeds [**74**]. The most common vegetable oils used for fish feed production have been soybean, linseed, rapeseed, sunflower, palm oil and olive oil. Plant oils, especially soybean and rapeseed oils, represent a source of oleic and linoleic acids and their production is considered sustainable in terms of water requirement since the wide-scale cultivation of these crops can be conducted in semi/arid regions [**75**]. However, the fatty acid profile of vegetable oil limits their use as FO replacer due to the lack of HUFA, especially DHA and EPA [**76**]. Rearing cultured fish species with high levels of vegetable oils respect to FO may reduce the PUFA content in aquaculture products with potentially negative marketing implications [**77**]. In fact, it has been demonstrated that partial (more than 50%) or complete replacement of FO with vegetable oil is feasible in practical diets without impairing fish growth, but significantly affects fillet fatty acid composition and metabolism [**78-81**].

Recently, developments in the genetic engineering have allowed the production of genetically modified plants ingredients which have been shown to possess more favourable features like the higher nutritional quality in terms of essential amino acids and fatty acid profile and the absence of ANF [**39**,**82**,**83**]. In particular, great attention has been given to the production of transgenic oilseed crops as source of long-chain PUFA to be used as FO replacers. On this regard, it has been demonstrated that it is possible to successfully engineered the crop *Camelina sativa* for the DHA and

EPA production by using constructs which encode the primary biosynthetic activities for n3 long-chain PUFA biosynthesis introduced into plants via A*grobacterium*-mediated floral transformation [**84**,**85**]. Transgenic plants producing HUFA could provide a sustainable alternative to FO able to sustain nutritional requirements of fish fed diets partially or completely deprived of marine-derived ingredients.

Microalgae and microbial biomass

Microalgae represent a promising solution to reduce the dependence on conventional raw materials (both marine- and plant-derived ingredients) in aquafeed formulation, highlighting several advantages in terms of their culturing and beneficial roles on fish overall welfare even at low dietary inclusion levels [**86**]. Microalgae can grow in a wide range of habitats with simple nutritional requirements, with biomass production rates several-folds higher than plants, can accumulate useful metabolites, and can be used, in microalgae-assisted aquaculture, to convert nutrients in wastewaters in valuable biomass further exploited to produce aquafeeds [**87**,**88**]. Microalgae represent a great source of protein, lipid (particularly PUFA and HUFA), vitamins, and pigments and, due to their nutritional value, have been shown to positively affect fish growth, the composition of the edible portion, and disease resistance [**89**].

Microalgal protein can be addressed as plausible alternatives to FM because of their good quality and an aminoacidic profile rich in essential amino acids and comparable with that of other conventional protein sources [**90**]. Several studies have reported the utilization different species of microalgae as a supplementary protein sources or partial substitutes for FM protein in diets of various omnivorous and carnivorous fish species [**91-93**]. In particular, the cyanobacterium *Artrhospira platensis* (Spirulina) contains all the essential amino acids, a high protein level (~68% of its biomass), and possess active components like phycocyanin and β-carotene with strong anti-inflammatory and antioxidant properties [**94**]. When used as feed supplement in FM-based diets, *A. platensis* has been shown to decrease oxidative stress and improve immune responses in rainbow trout [**95**,**96**]. Furthermore, feeding rainbow trout with *A. platensis* supplementation (up to 5%) in diets containing high percentages of vegetable-derived ingredients improved gut histological structure and overall health [**97**]. It has been also demonstrated that *A. platensis* may act as a growth promoter in different fish species when included as feed additive (singly or in combination with probiotics) in diets containing variable levels of vegetable-derived ingredients [**98-101**]. In particular, Teimouri et al. [**102**] showed that the FM replacement at 10% with *A. platensis* in diets did not negatively affect the weight gain and the growth rate of farmed rainbow trout, confirming the potential role of this cyanobacterium in enhancing the nutrient absorption at gut level, especially lipid [**102-104**]. Another

species of the genus *Arthospira* (*A. maxima*) has been shown to replace high percentages of FM (up to 43%) without impair growth or feed intake in Tilapia (*Oreochromis sp.*) [**105**,**106**].

An important microalgal species is represented by *Tetraselmis suecica* which represents a potential source of carotenoids (mainly astaxanthin) [**107**], that are able to exert a beneficial effect on intestine morphology and can act against inflammation [**108-111**]. In addition, *Tetraselmis suecica* was able to replace 20 and 45% (provided singularly or in combination with *Tisochrysis lutea,* respectively) FM protein without adversely affecting growth performance, quality traits and gut digestive-absorption functions in European seabass (*Dicentrarchus labrax*) [**109**,**112**,**113**]. However, it should be pointed out that certain microalgae species (among which *T. suecica*) possess a relatively thick cell wall consisting by a complex networks of polysaccharides and glycoproteins that can affect the digestibility of protein and lipid [**114**,**115**]. Particularly, carnivorous fish do not have the capacity to digest non-starch polysaccharides [**116**]. On this regard, previous studies reported a reduction in feed digestibility in European sea bass and rainbow trout fed with diets with variable inclusion levels of *T. suecica*, probably due to the relatively thick cell wall of this microalga [**112**,**113**,**117**,**118**]. Furthermore, feeding studies using *Nannochloropsis* species as ingredient for salmonids have reported a reduction of growth and nutrient (particularly long-chain PUFA) digestibility as a result of the presence of complex indigestible carbohydrates in the microalga cell wall [**119**,**120**].

As regards the lipid content, microalgae are often rich in HUFA, particularly ARA, EPA and DHA, even if fatty acid composition depends on the species and its rearing conditions (light intensity, culture media, temperature, and pH) [**86**]. On this regard, several autotrophically and heterotrophically grown species have been considered for their high EPA and/or DHA contents, such as *Isochrysis* sp.*, Nannochloropsis* sp., and the Thraustochytrids *Schizochytrium* sp., *Thraustochytrium* sp., and *Aurantiochytrium* sp. [**121-123**]. Although referred as microalgae, Thraustochytrids are protists that have lost photosynthesis and they are able to store large amounts of HUFA in heterotrophic conditions. Particularly, *Schizochytrium* sp. is characterized by a high lipid content (55-75% in dry matter) and up to 49% DHA of total lipids [**124**]. The exploitation of this protist as an alternative lipid source in aquafeeds has been investigated in different farmed fish species like Atlantic salmon [**125**,**126**], sea bream [**127**], and channel catfish [**128**]. Full replacement of FO with *Schizochytrium* sp. have been shown to improve weight gain, feed conversion ratio and protein efficiency ratio in Nile Tilapia (*Oreochromis niloticus*) [**129**]. Furthermore, in salmonids, dietary *Schizochytrium* sp. has been shown to improve the retention efficiency of long-chain PUFA in the edible portion and to improve the fillet pigmentation since nutritional components provided by this protist, especially DHA and vitamin E, have been shown to facilitate the pigments intestinal uptake and to provide a strong antioxidant effect [**125**,**126**].

The above-mentioned results evidence that microalgae represent a suitable source of protein and PUFA for aquaculture species, but drawbacks and challenges of using this alternative ingredient to replace FM and FO are still evident. Firstly, the high production costs still limit their use in the aquaculture sector [**90**,**129**]. Nowadays, there is a large discrepancy in the global supply and purchase cost of microalgal biomass versus existing commodity animal feedstuffs. Improving new technologies such as specialized mass culture facilities using heterotrophic methods and affordable closed photobioreactors for culturing microalgal and microbial strains may partially solve the problem [**130**,**131**]. In fact, beside the availability of microalgal biomass at sufficient quantity with suitable price, the inclusion of this raw material in aquafeeds should be evaluated at species level due to potentially large variations in proximate composition and digestibility encountered among different strains and growing conditions [**86**]. Particularly, effective treatment to disrupt the cell wall and thus make the algal constituents accessible to fish digestive enzymes can be applied to improve the digestibility [**132**]. Furthermore, it should be considered the use of a combination of different microalgae species since it allows higher dietary inclusion levels compared to their use a single ingredient [**112**].

Terrestrial animal by-products

The use of terrestrial animal by-products is a common practice in aquaculture in light to formulate cost-effective feeds with high protein and lipid sources [**39**]. Terrestrial animal by-products contain a good balance of essential amino acids and good digestibility, with features similar to those of FM [**133**,**134**]. Furthermore, these raw materials are relatively free from ANF, result to be highly palatable to most fish species [**135**], and have a lower carbon footprint compared to FM or vegetable alternatives [**136**]. Despite these advantages, the aquafeed inclusion of terrestrial animal by-products is still limited by consumer acceptance constraints and a strict regulation on their use due to the risk of developing human diseases (European Commission, 2013). In fact, in the European Union, their use was prohibited from 1990 to 2000 due to the arising of the bovine spongiform encephalopathy in ruminants and then, in 2013, was allowed only for animal by-products derived from non-ruminant animals (Category 3) [**137**].

Clean and fresh non-ruminant animal blood can be used to produce blood meal, which is generally characterized by high crude protein level (85%), a crude fat level of 0.5-3%, and a favourable content of lysine and histidine [**26**,**39**]. Agbebi et al. [**138**] reported that blood meal can totally replace FM in diet for in African catfish (*Clarias gariepinus*) juveniles with no adverse effect on growth, survival and feed conversion. Processing method can significantly affect the digestibility of blood meal since increased heat application usually deteriorates haemoglobin and cause low palatability [**39**].

Similarly, the inclusion of feather meal in aquafeeds depends on production technology [**139**,**140**]. Feather meal usually derives from the poultry industry, and it has been tested as partial or full replacement of FM in several teleost species [**141**,**142**]. However, high dietary levels of feather meal have been shown to impair growth performances [**141**,**142**]. Furthermore, from a nutritional point of view, feather meal is low in certain essential amino acids including lysine, methionine, and histidine [**39**].

Among the terrestrial animal by-products, the most promising cost-effective ingredients for aquafeeds is the poultry by-product meal (PBM) which consist of round part of poultry waste including legs, necks, underdeveloped eggs, and a limited fraction of feathers [**39**]. PBM shows a high protein content (50-80% on dry mass), an energy content similar to that of FM, with a well-balanced amino acid profile and good palatability [**135**,**143**], key features for carnivorous fish diets [**144**]. On this regard, Sealey et al. [**145**] demonstrated that PBM can completely replace FM without negative effects on growth and disease resistance in rainbow trout. Recently, the potential role of PBM as partial replacer of vegetable protein (singly or in combination with *Hermetia illucens* meal) in practical diet intended for rainbow trout has been deeply explored highlighting a beneficial effect on fish growth, gut microbiota, and overall health of gut and liver [**146**,**147**].

It should be pointed out that the differences in dietary PBM utilization exist among fish species and are related to the quality and quantity of PBM used in diets formulation [**135**]. Generally, PBM shows very low PUFA content limiting its inclusion levels, especially in marine species [**145**], and a deficiency in some essential amino acid (lysine, methionine, and histidine). On this regard, reduced growth performances were highlighted in several fish species fed diets characterized by high levels of PBM inclusion respect to FM, attributable to a deficiency in both essential amino acids and essential fatty acids [**140**,**148-150**]. Furthermore, Rossi and Davies [**151**] demonstrated that high dietary PBM inclusion respect to FM could lead to a decrease in taurine content beyond a threshold level that cause a reduction in growth performances in Florida pompano (*Trachinotus carolinus*). Despite not generally considered an essential amino acid, the ability to synthesize taurine widely varies among fish species due to different activities of key-enzymes in the biosynthetic pathway [**151-153**]. Furthermore, quality and nutritional value of PBM can change depending on the included materials and on the processing (time and temperature of the cooking process) applied for the production [**135**]. For that reason, the improvement of quality due to better processing technologies allows high levels of FM replacement. The use of branded poultry products, which utilize stringent systems to monitor and maintain product quality during manufacture, can reduce variability in nutritional quality, they generally have higher costs. In light of the available literature, further studies

are highly needed to evaluate the potentialities of PBM in aquafeeds formulation as promising replacers of more conventional ingredients, both marine- and plant- derived ones [**137**].

Insects

Insects are considered one of the most promising cost-effective, sustainable and alternative ingredients able to replace FM and FO in fish diets. In fact, besides their presence in the natural diet of both freshwater and marine fish species [**154**], insects are characterized by a proper nutritional profile (especially in terms of amino acids, vitamins and minerals) [**155**], and an environmental-friendly rearing (low energy and water consumption and no arable lands needed) [**156**]. Furthermore, most of insects larvae can grow on low quality organic wastes reducing the final mass by 50%, the nitrogen and phosphorous waste by 30-50% and 60-70%, respectively, and the load of pathogenic bacteria [**157**,**158**]. Besides the re-use of the remaining organic substrate, the result of this efficient bioconversion process is an abundant amount of larvae or prepupae rich in proteins, lipids, minerals and vitamins [**158**,**159**]. Furthermore, many insects species from Lepidoptera, Diptera, Hymenoptera, Coleoptera, Trichoptera, Hemiptera, and Odonata also show antifungal activity and/or antibacterial peptides [**160**] that may increase the shelf-life of insect-containing feeds [**161**] and can have beneficial effects on fish gut health [**147**].

The protein content of insects depends on the species and on the processing methods and ranges between 50 and 82% on dry matter [**159**,**162**], comparable to those observed for the conventional aquafeeds ingredients (up to 73 and 50% for FM and soybean meal, respectively) [**163**]. In addition, the amino acid profile of most insect species tested in fish diet meet the fish requirements [**26**,**164**]. It should be pointed out that the amino acid profile of insects is taxon-specific. Diptera have been shown to possess an amino acid profile close to that of FM, while Lepidoptera and Hymenoptera deserve particular interest due to their richness in methionine [**159**,**165**,**166**]. Many species of locusts, houseflies, and mealworms but also the honeybee *(Apis mellifera*) and the common fruit fly (*Drosophila melanogaster*) are rich in taurine and hydroxyproline that positively affect fish growth and health [**165**]. Differently, Coleoptera and Orthoptera evidenced similarities with the soybean meal profile, with potential deficiencies of lysine and methionine [**163**]. Dietary deficiencies in essential nutrients may affect fish growth and feed conversion rate with a consequent appearance of deficiency symptoms and then sensitivity to diseases [**167**,**168**]. In order to better fit fish dietary requirements, the use of a mixture of different protein sources, like other animal proteins, can reduce the nutrient deficiencies and better balance the amino acid profile of insect-based diets. On this regard, Randazzo et al. [**147**] demonstrated that a combination of black soldier fly (*Hermetia illucens*; BSF) prepupae meal and PBM led to the best overall response in terms of growth and gut and liver health in rainbow

trout (*Oncorhynchus mykiss*) respect to provide the single ingredients singly. Furthermore, combining superworm (*Zophobas morio*) meal with a 10% of the mushroom *Pleurotus sajorcaju* significatively improve the zootechnical performances of Nile tilapia (*Oreochromis niloticus*) possibly due to a better amino acid balancing and the mushroom prebiotic properties [**169**,**170**].

Considering lipids, the content in insects is generally higher than those observed in FM (8.2%) and plant-derived ingredients like soybean meal (3.0%) and ranged from 10 to 30% [**165**]. In particular, terrestrial insects have been shown to be rich in SFA and rather than in long-chain PUFA and their dietary inclusion may impair fish growth and welfare, especially in marine fish species [**40**,**171**,**172**]. Furthermore, the use of insect-based diets that can possibly affect fillet fatty acid composition leading to a reduction of HUFA-related health benefits for human consumers and to flavour and aroma modifications [**165**]. Given the inadequate fatty acids composition of insect meals that can limit their inclusion in aquafeeds, the lipid fraction can be separated by the high-protein insect meal through a defattening process and can be used in biofuels [**173**,**174**]. However, this procedure represents an important cost in insects manufacture and it has been demonstrated that, in Atlantic salmon (*Salmo salar*), highly defatted BSF, dried at a conventional temperature reduced fish growth compared to fish fed lightly defatted BSF dried at a low temperature [**165**]. It should be pointed out that a high variability in insects fatty acids profile among different species, life-cycle stages and rearing substrates have been reported [**163**]. Particularly, it has been demonstrated the insects' fatty acid profile can be strongly modulated by the rearing substrate rich in PUFA itself or enriched with potential sources of PUFA. For example, partly (25 and 50%) feeding BSF prepupae with fish offal for a month led to a 25-50% increase of FM replacement in diets intended for rainbow trout (*Oncorhynchus mykiss*) without affecting growth [**175**]. Differently, Truzzi et al. [**176**] demonstrated that culturing BSF larvae on an organic substrate enriched with 10% *Schizochytrium* sp. significantly increased the PUFA content of insects.

Besides the good potential of insects' nutritional profile, one of the most controversial features related to the inclusion of insect meal in aquafeeds is the presence of chitin. Chitin is a mucopolysaccharide polymer consisting of β 1,4-linked N-acetyl-D- glucosamine residues which is encompassed in a matrix of proteins, lipids and other compounds to form the cuticle of insects [**177**]. It is commonly assumed that monogastric animals, including fish, cannot digested chitin [**178**], which, in turn, may induce a general reduction nutrient digestibility and assimilation [**179**,**180**]. The digestion process requires chitinase, chitobiase and lysozyme that have been found to be present in both freshwater and marine carnivorous and omnivorous fish [**181**]. However, the presence of chitin-derived matrix in fish gut may reduce the access of chitinases and proteinases to their substrates and may prevent the absorption of proteins and lipids by the intestinal mucosa. In this way, both chitin and proteins and

lipids digestibility is reduced, leading to a worsening in nutrient utilization and fish growth performances [**165**]. On this regard, the dietary inclusion of high percentages of BSF prepupae meal in rainbow trout (*Oncorhynchus mykiss*) and carp (*Cyprinus carpio*) diet also induced intestine morphology changes and consequent effects on growth [**182**,**183**]. However, it has been demonstrated in several fish species that moderate inclusion of chitin resulted in immunity boosting [**184**,**185**] and positive microbiota modulation [**186**,**187**]. In particular, chitin represents one of the main growth substrates of lactic acid bacteria [**185**,**187**] that are crucial in making available indigestible carbohydrates and contribute to the synthesis of vitamins and short-chain fatty acids, considered as the primary energy source for enterocytes [**188**], or of important anti-inflammatory molecules, like butyrate [**189**,**190**].

Insects as alternative ingredient for aquafeeds formulation have been firstly investigated in herbivorous/omnivorous fish. Over the last decade, interest has increased, and several studies have been conducted on different fish species, including carnivores one, mainly using insects from Orthoptera, Coleoptera and Diptera orders.

Orthoptera order includes locusts, grasshoppers and crickets which are highly nutritious, especially in the adult phase [**191**]. It has been demonstrated that a 13-25% dietary inclusion of adult Orthoptera did not affect the diet digestibility and fish growth in both African catfish (*Clarias gariepinus*) and Nile tilapia (*Oreochromis niloticus*) [**164**,**165**]. A reduction of growth was evidenced only as a result of a total FM replacement with the variegated grasshopper (*Zonocerus variegatus*) in diets intended for both African catfish (*Clarias gariepinus*) due to an impairment of protein and lipid digestibility and to arginine and lysine deficiencies [**164**].

Among Coleoptera species, the superworm (*Zophobas morio*) represent a rich source of protein and contains adequate quantities of the essential amino acids required for an optimal fish growth [**163**], but is poor in minerals like calcium and phosphorous [**192**]. The 25 and 50% of FM replacement with of superworm meal resulted in an optimal growth of Nile tilapia (*Oreochromis niloticus*), while a 75% of FM replacement was well tolerated, in terms of growth, feed efficiency and protein digestibility, and showed no significant difference from FM-based diet [**193**]. Differently, the yellow mealworm (*Tenebrio molitor*) has received great interest due to the high protein and lipid content of the larvae that can be provided live or as a meal [**165**]. This insect species showed positive results in the diets of both freshwater and marine fish. Yellow mealworm larvae have been shown to replace up to 60% of dietary FM without negatively affect growth and feed utilization in African catfish (*Clarias gariepinus*), while higher FM replacements (80 and 100%) decreased fish growth performances [**194**]. Promising results have been obtained also in rainbow trout (*Onorhynchus mykiss*) in which the efficacy of yellow mealworm as promising alternative to FM in practical diets

for fry (including 7, 14, 21, and 28% of insect meal respect to FM) has been proved not only in relation to growth rates and feed utilization, but also in terms of immunopotentiation effects [**195**]. Furthermore, no major effects of a total FM substitution with yellow mealworm meal on bacterial species richness and diversity in both gut mucosa- and skin mucus-associated microbiome were evident [**196**]. Considering marine fish species, *Tenebrio molitor* larvae have been successfully used in gilthead seabream (*Sparus aurata*) and European sea bass (*Dicentrarchus labrax*), where 25% of FM replacement did not impair fish growth [**165**]. However, in these studies, a 50% FM replacement levels significatively reduced growth of both these marine fish, and European sea bass showed a reduced n3 HUFA concentration in the fillets [**165**]. More recently, it has been demonstrated that it is feasible to substitute dietary FM by defatted yellow mealworm up to 80% in European sea bass (*Dicentrarchus labrax*) without detrimental effects on nutrient digestibility, growth performance and associated genetic pathways, whilst assuring fillet nutritional value for human consumption [**197**].
Finally, FM replacements have also been investigated using meals obtained from two species of Diptera, the common housefly (*Musca domestica*) and the BSF (*Hermetia illucens*). Due to their ubiquity, common housefly larvae have been extensively tested in fish diets, but results are controversial. Fish growth improvement have been demonstrated in Nile tilapia (*Oreochromis niloticus*) and black carp (*Mylopharyngodon piceus*) fed different inclusion levels of common housefly larvae meal [**198**,**199**], while a worsening in zootechnical performances have been evidenced in African catfish (*Clarias gariepinus*) and rainbow trout (*Oncorhynchus mykiss*) [**200**,**201**]. Differently, promising results have been obtained in several fish species using BSF larvae or prepupae meal and will be extensively reviewed in Chapter 5. In fact, in the last years, the BSF has been recognised as one of the most promising alternatives to conventional marine-derived ingredients due its nutritional profile, the environmental-friendly rearing and the possibility to introduce and promoting circularity in the aquaculture sector exploiting its bio-converting ability.

1.4

The Black Soldier Fly as one of the most promising candidates for aquafeed formulation

The BSF (*Hermetia illucens* Linnaeus 1758, Diptera, Stratiomyidae family) is a fly native form the tropical, subtropical and warm temperate areas of America, but the development of international transportation since 1940s led to its spread in tropical and warmer temperate world regions between about 45° N and 40° S [**191**]. BSF larvae can feed from 25 to 500 mg of fresh matter/larva/day on a wide range of decaying organic substrates like rotting fruits and vegetables, cow manure, fish offal, brewery by-products, restaurant waste and sewage sludge [**202**,**203**,**205**,**206**]. In ideal conditions, BSF larvae become mature in 2 months reaching up to 27 mm in length and 6 mm in width and weigh up to 220 mg; differently, the larval stage can last up to 4 months if not enough feed is available. At the end of the larval stage (prepupa), the BSF empties its digestive tract and stops feeding and then migrate in search of a dry and protected pupation site. The duration of the pupal stage can be extremely variable, normally lasting 14 days, but extending up to 5 months. The adult is black, wasp-like and 15-20 mm long do not feed and rely on the fats stored from the larval stage. Females mate two days after emerging and oviposit into dry cracks and crevices adjacent to a feed source [**191**]. A year-round breeding adult colony needs to be maintained in a greenhouse with access to full natural light throughout the year. The greenhouse must be a minimum of 66 m^3 to allow for the aerial mating process and optimal temperature and humidity ranges to ensure mating oviposition must be from 24-27.5 °C to 37.5-40 °C and from 30-50 to 90%, respectively. The greenhouse needs a container with an attractive and moist medium to attract egg-laying female adults [**207**].

Rearing facilities exploit the migrating behaviour of the prepupae for self-collection: larvae climb up a ramp out of a rimmed container to eventually end in a collecting vessel attached to the end of the ramp [**191**]. Optimum rearing conditions include a narrow range of temperature (between 29 and 31 °C), humidity (between 50 and 70%) and suitable levels of texture, viscosity, and moisture content of the substrate. In fact, higher relative humidity than 70% makes the substrate too wet for larval migration and for an adequate oxygen supply [**207**]. The BSF rearing procedure is considered sustainable in terms of land use, water consumption and CO_2 production, because of their low environmental requirements [**208**,**209**]. However, the rearing facility requires a warm environment which may be energy-consuming to sustain in temperate climates. BSF is able to resist to extremely

adverse environmental conditions like drought, food shortage or oxygen deficiency, but temperature and substrate quality and quantity deeply affect the duration of the life cycle ranging it between several weeks to several months [**191**].

Since 1990s, the BSF rearing has been proposed as an efficient method to processing organic wastes by converting them into valuable biomass rich in proteins and lipids, suitable for animal feeding and biodiesel and chitin production [**155**,**191**]. Dense population of BSF larvae can convert large volumes of organic waste into valuable biomass, solving a number of environmental problems associated with manure and other organic wastes [**155**]. It has been demonstrated that BSF larvae can reduce laying hen or pig manure accumulation by 50% or more without using extra resources including energy [**158**,**207**], while in confined bovine facilities, they were found to reduce available phosphorous and nitrogen by 61-70% and 30-50%, respectively [**191**]. BSF larvae are able to quickly process organic substrates with high bio-conversion efficiency, reducing bacterial growth and aerating and drying the substrate, reducing odours [**155**]. This insect species is a competitor of the common housefly (*Musca domestica*) larvae, as they make manure more liquid and thus less suitable for housefly larvae and for their oviposition, reducing the whole population by 94-100%. As a result, they can help to control housefly populations in livestock farms and in households with poor sanitation, thereby improving the health status of animals and people since housefly is a major vector of diseases [**158**]. On this regard, unlike other fly species, the BSF is not a disease carrier since the adult fly cannot eat due to its lack of functioning mouthparts [**155**]. Furthermore, BSF larvae modify the manure microflora, potentially reducing harmful bacteria such as *Escherichia coli* and *Salmonella enterica* [**210**]. Finally, BSF adults are not attracted to human habitats or foods and are not considered a nuisance [**155**].

Because of their nutritional composition, BSF larvae have been used as an ingredient in feed for various animals like poultry and fish [**191**]. In fact, the current legislation (Reg. 2021/1372) in Europe allows the use of this innovative ingredient within aquaculture and for non-ruminant farmed animals [**204**]. BSF larvae represent a high-value feed source that contains about 40-44% of crude protein and an amino acid profile like that of FM. Particularly, lysine is abundant representing 6-8% of the total protein. The dry matter content of fresh larvae ranges between 35-45% and makes them easier and less costly to dehydrate than other fresh by-products [**191**,**211**]. Ash content is relatively high but variable (11-28% on dry matter) as well as calcium (5-8% on dry matter) and phosphorous (0.6-1.5% on dry matter) [**191**]. The amount of fat is extremely variable and is strictly related to the rearing substrate type [**158**,**191**]. As a consequence, great similarities have been evidenced between the fatty acids composition of the larvae and the substrate [**171**]. Besides this dependency to the rearing substrate, BSF larvae have been found to contain a high amount of SFA in comparison to other insects species [**212**]. Since BSF adults do not feed, the high SFA content of the larvae represents a way to

store energy for the later life stage since these fatty acids are less prone to oxidation than unsaturated ones [**213**]. Among SFA, lauric acid (12:0) is particularly abundant in BSF larvae which synthesise it from the carbohydrates in the substrate independently from its composition [**205**]. This fatty acid represents a key feature in the use of BSF in aquafeeds formulation since it has been demonstrated to have beneficial effects on fish gut welfare by mitigating inflammatory conditions due to its anti-inflammatory, anti-bacterial and anti-viral properties [**214**,**215**].

BSF larvae are not able to synthesise long-chain PUFA and fatty acids of particular interest for aquafeeds formulation like linoleic acid, α-linolenic acids, EPA and DHA found in larvae most derived from the substrate [**171**,**214**]. On this regard, feeding BSF larvae with a rearing substrate made of organic by-products containing desirable n3 fatty acids is therefore a way to enrich the final biomass [**175**,**206**]. Furthermore, it is possible to tailor the larval fatty acids profile, especially in terms of EPA and DHA, by enriching the rearing substrate with external sources of PUFA [**214**]. Recently, Truzzi et al. [**176**] demonstrated that culturing BSF larvae on an organic substrate made of coffee silverskin enriched with 10% *Schizochytrium* sp. significantly increased the PUFA content of the final biomass.

In light of the further development of the aquaculture sector, BSF larvae and prepupae represent an innovative, more sustainable and cost-effective solution to face the problems related to the more conventional ingredients (both marine- and plant-derived ones) in aquafeeds formulation. Their high bio-conversion efficiency can be exploited to convert organic by-products into valuable biomass allowing to introduce circularity in the aquaculture sector, interconnecting land and oceans.

1.5

Fish welfare in aquaculture

The welfare of farmed fish species has received great attention due to the rapid expansion of the aquaculture sector [**216**]. In fact, fish welfare represents a key point for this industry not just for public perception, marketing and product acceptance, but also to ensure production efficiency, quality and quantity [**217**]. The provision of high amounts of fish to satisfy the increasing consumer's demand forces aquaculture to high production rates [**2**] that not always ensure a proper fish welfare. Animal welfare has been defined has the ability of an animal to adapt to its environment and remain in good health, to live a natural life and express its natural behaviour, and/or an animal's subjective mental state [**216**,**218**]. This concept has been traditionally applied to species considered to have the ability to experience pain, fear and suffering and, thus, with a higher level of cognition compared to fish. However, the possibility that fish possess the cognitive and behavioural complexity to experience feelings and suffering is now a much debated topic within research community [**219**,**220**]. In light of this, current European legislation assumes that fish are sentient beings capable to suffer [**218**].

In aquaculture, stressors are unavoidable but reducing stress and its harmful effects represents one of the main goals to combine successful production with fish growth and welfare. Fish behavioural and physiological stress responses are commonly used as indicators of compromised welfare [**216**,**221**]. In addition, molecular biomarkers directly indicating gene activity may have the characteristics for being useful early indicators. A biomarker is defined as any biological response (ranging from molecular through cellular and physiological responses to behavioural changes) to a stress factor measured inside an organism leading to a deviation from the physiological state that cannot be detected in the intact organism [**222**]. In order to assess the exposure of fish to environmental stress conditions, several biomarkers should be examined to obtain a comprehensive response.

In teleosts, stress activates the hypothalamus-pituitary-interrenal (HPI) axis, leading to a rapid release into the blood stream of adrenocorticotropic hormone (ACTH) and a subsequent cortisol secretion by the interrenal tissue, analogous to the adrenal cortex in mammals [**223**]. The effects of cortisol in fish consist in mediating stress-induced hyperglycemia, which is crucial for supporting the stress-associated increased energy demand, through activation of phosphoenolpyruvate carboxykinase (PEPCK) and gluconeogenesis [**224**]. Cortisol released into the circulatory system enters cells by passive diffusion or is facilitated by a carrier-mediated process [**225**]. Inside the cell, it binds to a

high-affinity cytosolic glucocorticoid receptor (GR), which acts as a ligand-dependent transcription factor to control and regulate gene expression [**226**], interacting with glucocorticoid response elements (GREs) or with numerous cytosolic proteins including chaperones, kinases, phosphatases and proteasome [**227**]. Receptor number or affinity may directly influence the degree of reactivity of target cells [**228**]. The activation of HPI results in energy source mobilisation, depletion of glycogen stores, and an increase in plasma levels of glucose, along with high muscle activity, anaerobic glycolysis and an increase in plasma lactate. This triggers a suite of secondary responses (*i.e.* heightened cardiorespiratory activity, splenic release of red blood cells and mobilization of energy stores), which serve adaptive functions for individual's experiencing demanding conditions in nature [**229**]. However, prolonged and/or repeated stress imposed on fish may result in detrimental tertiary stress responses which negatively affects appetite, growth, swimming ability, immune responses, behaviour and reproductive performances [**216**,**221**,**230**]. Furthermore, chronic stress exerts a strong effect on haematology [**231**], metabolism [**232**], neuroendocrine function [**233**], and hydromineral balance and osmoregulation [**234**]. The tertiary stress responses have been commonly used as indicators for compromised welfare of fish in aquaculture, since it is indicative of prolonged, repeated or unavoidable stress factors [**229**]. Measures stress markers gene expression is able to provide useful and sensitive biomarkers to diagnose stress and improve fish welfare [**235**,**236**].

At cellular level, the stress response is mediated by the heat shock proteins (HSP), a family of highly conserved proteins that are present in all cells in all life forms [**237**]. In fish, HSP are robustly induced by diverse stressors that denature proteins, including perturbations of temperature and salinity, environmental contaminants, handling, hormones and biotic stressors.

Stress is, therefore, a major factor in the health and welfare of farmed fish. Farmed fish are usually exposed to a variety of stressors including handling, stocking density, and nutrition which long-term exposure could have negative effects on welfare and growth performances [**40**,**229**,**238**,**239**]. In addition, all these stressors can strongly increase susceptibility to naturally occurring pathogens. Besides optimal fish maintenance procedures, proper aquafeed formulations are of primary importance to sustain high fish production and standards without compromise fish welfare [**3**]. In light of the search of marine- and plant-derived ingredients substitutes, when novel aquafeed formulations are tested, monitoring integrity of the gastrointestinal tract, and more generally the integrated physiological response, become fundamental to assess their suitability for aquaculture sector. In fact, the gastrointestinal tract is involved in the digestion and absorption of nutrients as well as in the innate and adaptive immunity of fish which can both be deeply modulated by the diet [**240-242**]. Furthermore, liver plays a key role in many fish metabolic pathways and its morphological

structure and macromolecular composition are deeply influenced by the diet [**41**,**243**,**244**], deserving a special attention when fish welfare is evaluated.

Since malnutrition is nowadays considered a stressor [**245-247**] the investigation of the health status of both gastrointestinal tract and liver in response to new aquafeed formulations represents a key point for the maintenance of optimal fish rearing condition.

1.6

Aim of the thesis

In an ever-expanding aquaculture industry, aquafeed formulation has been identified as one of the key factors challenging the long-term ecological and economical sustainability of this sector [**2**,**248**]. In light of this, several efforts have been done over the last decades to fully or partially substitute unsustainable marine- and plant-derived ingredients with alterative, cost-effective and equally healthy ones derived from both vegetables and animals [**249**,**250**]. Among all the alternatives proposed in the last decades, insects and, particularly, the BSF have represented one of the most promising aquafeed ingredients due to an environmental-friendly rearing and a proper nutritional profile, especially in terms of protein content and amino acid profile [**191**]. Furthermore, BSF contains important bioactive molecules like chitin and lauric acid with immune-boosting properties that can exert beneficial effects on fish gut health [**185**,**215**]. The main bottleneck in the use of BSF in aquafeed formulation is still represented by the fatty acids profile, rich in SFA rather than in PUFA that are fundamental for a proper fish growth and welfare as well as for fillet macromolecular composition in light of human nutrition [**40**,**171**].

The aim of the present thesis is investigating the physiological effects of different dietary inclusion levels of BSF prepupae meal firstly on an experimental model (zebrafish; *Danio rerio*) considering the whole life cycle and the possible impact across generations and then focusing on a freshwater (Siberian sturgeon, *Acipenser baerii*) and a marine (gilthead seabream, *Sparus aurata*) farmed fish species. Applying a multidisciplinary approach involving biometric, histological, molecular, gas chromatographic, and spectroscopic analyses, particular emphasis will be given on fish growth, fillet fatty acids composition and liver/gut health.

In the first section (Chapter 2), a control FM-based diet and two BSF-based diets characterized by 25 and 50 % of full-fat BSF prepupae meal inclusion respect to FM were tested during the whole life cycle of zebrafish, from larvae to adult, in order to assess the physiological effects on fish growth and welfare (Chapter 2.1) as well as on reproductive performances of female specimens (Chapter 2.2).

The second section (Chapter 3) proposes new BSF-based diets built on the circular economy concept and formulated using BSF prepupae reared on coffee industry by-products (coffee silverskin) enriched with 10% of *Schizochytrium* sp. (Thraustochytriaceae) as a source of PUFA to improve insects' final biomass. Thraustochytrids are recognised are group of marine oleaginous protists able to synthesise large amount of HUFA [**251**]. A control FM-based diet and four BSF-based diets

characterized by 25, 50, 75 and 100 % of full-fat “enriched” BSF prepupae meal inclusion respect to FM were tested during zebrafish larval development (Chapter 3.1), in zebrafish juveniles (Chapter 3.2) to assess fish growth and welfare as well as in adult zebrafish focusing on reproductive performances of mature females (Chapter 3.3). The same diets were then further tested on the larval development of the first filial generation to investigate possible dietary adaptations mechanisms across generations (Chapter 3.4).

In the third section (Chapter 4), the physiological effects of BSF-based diets on farmed fish species were analysed. Since only fragmentary information is available about the use of BSF-based diets in Siberian sturgeon nutrition and considering the circular economy concept and a more sustainable aquaculture development, a 60-day feeding trial was conducted representing the first comprehensive multidisciplinary study on the physiological effects of a 50% dietary inclusion of “enriched” BSF prepupae meal respect to FM during sturgeon culture in an aquaponic system (Chapter 4.1). Furthermore, since the attempt to replace marine-derived ingredients for aquafeeds formulation with plant-derived ones has met some limitations due to their negative side effects on many fish species, two promising alternatives, PBM and BSF prepupae meal, were used, singly or in combination, to replace the vegetable protein fraction in diets totally deprived of FM intended for gilthead seabream in a 12-week feeding trial (Chapter 4.2).

Finally, the last section (Chapter 5) explore how some of the most promising laboratory approaches like histology, infrared spectroscopy, gut microbiome sequencing, molecular biology, fish fillets’ physic, chemical and sensory properties have been essential to assess fish welfare and fillet quality when BSF has been used as aquafeed ingredient in different diets in terms of BSF dietary inclusions, developmental stage (larvae or prepupae) and lipid content (full-fat, partially or totally defatted) on a wide range of fish species, with particular emphasis on European finfish species and experimental models.

1.7

Summary table of the studies

		Experimental model	Life cycle stage	Dietary treatments	Aim of the study
Chapter 2	2.1	zebrafish	adults 6 months dpf	0, 25 and 50 % of full-fat BSF prepupae meal respect to FM	Integrating biometric, histological, molecular, gas chromatographic and spectroscopic analyses to better evaluate **fish growth and welfare** in response to BSF-based diets administration during the whole life cycle.
	2.2	zebrafish	adult females 12 months dpf	0, 25 and 50 % of full-fat BSF prepupae meal respect to FM	Investigating the effects of BSF-based diets on **reproduction** in female specimens through the evaluation of growth, gonadosomatic index, spawned/fertilized eggs and hatching rate, fatty acids composition of adult females' carcasses and fertilized egg, histological analysis of the ovary, spectroscopic macromolecular composition of class IV oocytes, and expression of genes involved in fish lipid metabolism in the liver.
Chapter 3	3.1	zebrafish	larvae 21 dpf	0, 25, 50, 75 and 100 % of "*Schizochytrium*-enriched" full-fat BSF prepupae meal respect to FM	Diets were used for a feeding trial during larval development to assess **fish growth and welfare** through biometry, histology, gas chromatography, spectroscopy (FTIR), microbiota and molecular analyses.
	3.2	zebrafish	juveniles 2 months dpf	0, 25, 50, 75 and 100 % of "*Schizochytrium*-enriched" full-fat BSF prepupae meal respect to FM	Diets were used for a feeding trial until the reaching of juvenile stage to assess **fish growth and welfare** through biometry, histology, gas chromatography, spectroscopy (FTIR), microbiota, behavioural and molecular analyses.
	3.3	zebrafish	adult females 6 months dpf	0, 25, 50, 75 and 100 % of "*Schizochytrium*-enriched" full-fat BSF prepupae meal respect to FM	Investigating **growth, stress response, lipid metabolism and reproductive performances** in adult females. A multidisciplinary approach including biometric, gas-chromatographic, histological and molecular analyses was used to obtain a comprehensive overview of fish responses to the test diets.
	3.4	zebrafish	F1 generation 21 dpf	0, 25, 50, 75 and 100 % of "*Schizochytrium*-enriched" full-fat BSF prepupae meal respect to FM	Investigate, for the first time, whether **nutritional programming** exists in zebrafish larvae fed diets including increasing dietary levels of full-fat BSF prepupae meal, with emphasis on fish growth, health and fatty acids composition.

Chapter 4	4.1	Siberian sturgeon	juveniles 60-day-trial	0 and 50 % of "*Schizochytrium*-enriched" full-fat BSF prepupae meal respect to FM	**Zootechnical performances, fillet fatty acids composition, liver and gut integrity**, expression of genes involved in fish growth, stress and immune response and gut microbiome were investigated representing the first multidisciplinary study on the physiological effects of BSF-based diets in sturgeon juveniles and the first feeding trial using insect-based diets performed in an **aquaponics system**.
	4.2	gilthead seabream	juveniles 12-week-trial	- A diet rich in plant-derived ingredients, named CV. - A diet rich in fish meal, named CF as a positive control. - Five diets, named H20, H40, P20, P40, and H10P30 in which graded levels (20 or 40 %) of crude protein from the mixture of vegetable protein sources of the CV diet were replaced with crude protein from a commercial partially defatted BSF prepupae meal and/or poultry by-product meal (PBM) singly or in combination.	Investigating **growth performance and gut and liver health** in response to **diets without fish meal** through biometric, histological, spectroscopic and molecular analyses.
Chapter 5	5.1	-	-	-	Exploring some of the most promising laboratory approaches like histology, infrared spectroscopy, gut microbiome sequencing, molecular biology, fish fillets' physic, chemical and sensory properties, essential for a better understanding of fish welfare and fillet quality, when BSF is used as aquafeed ingredient. In particular, great importance has been given to European finfish species and experimental models.

Abbreviations: dpf days post fertilization

1.8

Model organisms

Zebrafish

Zebrafish (*Danio rerio*; Figure 1) belongs to the Cyprinidae family, composed of more than 2000 species, and to the Rasborinae subfamily [**252**]. It is a freshwater fish that evolved in South Asia around 320 million years ago, and it lives in tropical areas subjected to typical monsoon climate fluctuations inhabiting rivers, small streams and other channels, stagnant or slow-moving pools near streams and rice paddies [**253**]. Due to the wide range of habitats in which it can live with considerable physical-chemical variations, zebrafish shows a high adaptability. In fact, it is considered an eurythermic species, tolerating temperatures ranging from 16.5 to 38.6 °C [**254**], and different acidities, with pH values ranging from 5.5 to 9 [**252**]. The water physical-chemical properties under laboratory controlled conditions may differ from those found in the wild and maintained at optimal values (temperature: 26-28 °C; pH: 7-8) [**255**].

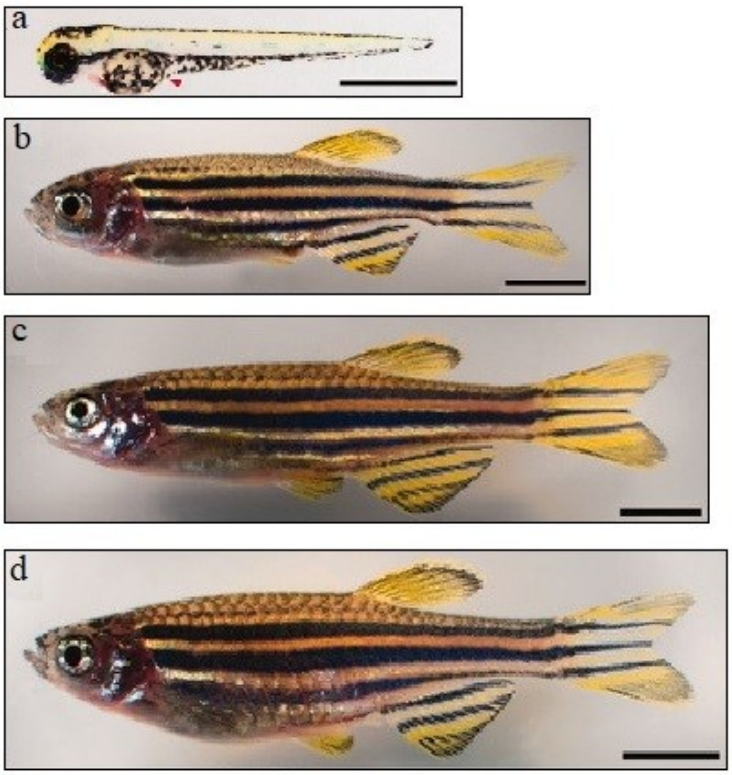

Figure 1. Different life cycle stages of zebrafish. (**a**) larvae; (**b**) juvenile; (**c**) adult male, (**d**) adult female. Scale bars: (**a**) 1 mm, (**b**) 3 mm, (**c**,**d**) 4 mm. Picture from Parichy et al. [**256**].

Zebrafish is considered euryphagous omnivore. In the wild, zebrafish diet is mainly based on zooplankton and insects, even if, based on gut content analysis, phytoplankton, filamentous algae, and invertebrate eggs, may also be included [**257**]. In captivity, each life cycle stage requires an appropriate type of food, starting from rotifers, *Artemia salina* nauplii and formulated microparticle diets during the larval development and dried feeds for juveniles and adults [**258**]. Adult zebrafish feed primarily in the water column and, in contrast to the wild, under laboratory conditions zebrafish do not seek the feed on the tank bottom, so overfeeding, and thus, excess of uneaten food can reduce water quality [**257**]. Considering the anatomy of the digestive tract [**259**], zebrafish intestine consists of one long tube that folds twice in the abdominal cavity. The intestine starts with a wide lumen that progressively becomes smaller in a rostral-to-caudal direction. No stomach, small intestine, or large intestine can be distinguished, but differences can be found in the morphology of the mucosa columnar epithelial cells and the number of goblet cells, suggesting functional differentiation (Figure 2). Columnar-shaped enterocytes are the most numerous cells in the zebrafish intestinal epithelium, followed by goblet cells, and are responsible for nutrient absorption. Like in mammals, an important role in the metabolic homeostasis of the body, including processing of dietary-assumed carbohydrates, proteins, lipids, and vitamins, is played in fish by the liver. The zebrafish liver includes three lobes that lie along the intestinal tract (Figure 2), differing from mammalian liver by the fact that hepatocytes are not clearly organized in cords or lobules, the typical portal triads are not apparent, and it does not have Kuppfer cells. Furthermore, mature females' hepatocytes are very basophilic due to the vitellogenin production.

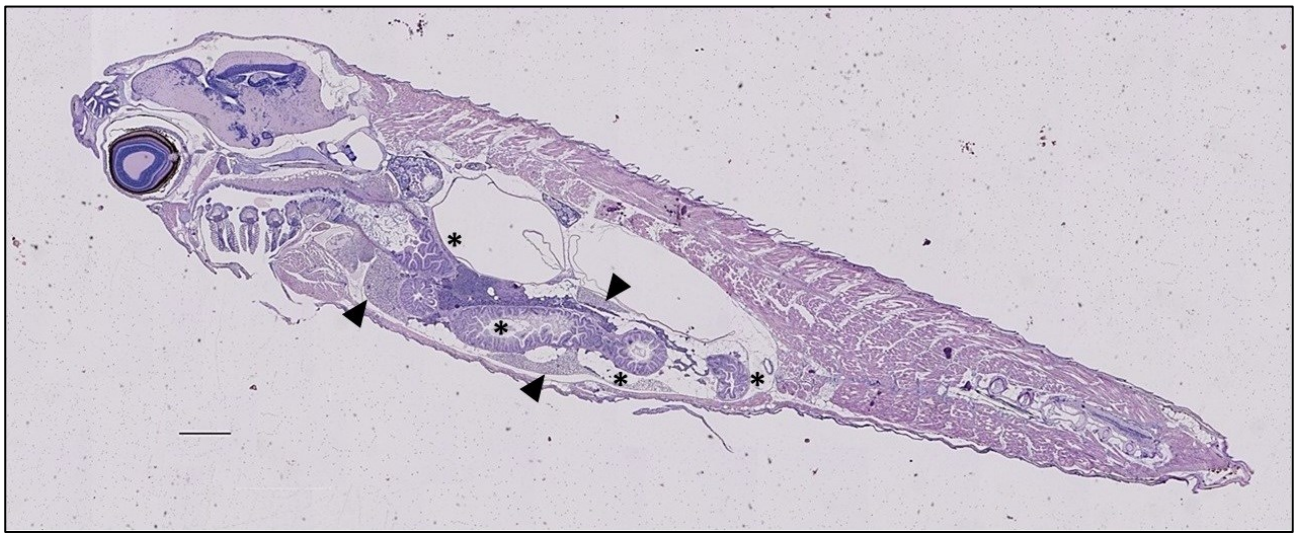

Figure 2. Histological section of a whole juvenile zebrafish. Scale bar: 500 µm. Asterisks identify different gut tracts, while arrowheads highlight different liver portions that lie along the intestine.

Zebrafish has a much shorter life cycle respect to most of the farmed fish species, reaching reproductive competence at 3 months [**252**]. Zebrafish exhibit a sexual growth dimorphism based on

differences in body-size, shape and pigmentation, easier to spot in adult specimens. Males are usually smaller and have a more elongated body with gold and blue stripes, whereas females are bigger, more rounded with a whitish belly, silver and blue stripes and exhibit a small genital papilla in front of the anal fin [**256**]. Females are asynchronous fish, with oocytes at different maturation stages present at the same time in the ovaries [**260**]. In the wild, zebrafish are mainly annual breeders and the spawning season starts just before the monsoon (August), at a time of high temperatures and food availability [**261**], while under laboratory conditions eggs are spawned throughout the year [**260**] with the best spawning performance every 10 days [**262**]. Females produce in one clutch several hundred eggs and, under laboratory conditions, female clutch size is larger than in the wild [**252**,**257**]. Within the same clutch, hatching occurs during the first 3 days [**263**]. The larval stage lasts up to 14-20 days (approximately 5 mm of total length) before the onset of metamorphosis which involves the larval fins absorption, the gut tube shift to a more ventrally position and the start of the scales development and the pigmentation patterns [**264**]. Juvenile fish appear during the 4th week of development, when the pigment dorsal pattern is formed [**265**]. Wild and domesticated zebrafish appear to reach reproductive maturity at similar sizes, usually at around 3-4 months of age [**266**].

Zebrafish is a widely used experimental model in several research fields like developmental biology, neurobiology, molecular genetics and toxicology [**267**]. Over the last decade, it has been recognised as a useful model organism for fish nutrition studies to be applied in the aquaculture sector [**252**,**267**,**268**]. A good fish model for aquaculture research must fulfil four key conditions: (i) possessing the same basic biological features of the most important cultured species; (ii) showing similar physiological responses to the most important cultured species, (iii) having a short life cycle and being easy and inexpensive to breed; (iv) having many resources (strains, genomic resources, lines and mutants) that facilitate research in most areas. It has been demonstrated that zebrafish, more than any other species, fulfils to a large extent all these conditions [**252**,**267**,**268**]. It shows several advantages that include the small size, the short generation time, the capacity to produce numerous offspring, the ease in breed and maintenance [**269**]. Furthermore, zebrafish have a wide variety of molecular tools and information available for genomic analysis, and the most powerful genomic resource in zebrafish is the accessibility of its complete genome sequence [**270**]. Furthermore, a rapid development of genetic and genomics resources has been made during the last decade for economically important farmed fish species such as channel catfish (*Ictalurus punctatus*) [**271**] rainbow trout (*Oncorhynchus mykiss*) [**272**], Atlantic salmon (*Salmo salar*) [**273**], Atlantic cod (*Gadus morhua*) [**274**] and gilthead sea bream (*Sparus aurata*) [**275**]. Within the ray-finned fishes (Actinopterygii), the dominant group today is the teleosts which include important farmed fish species [**269**]. Their evolutionary divergence and extreme diversity provide an abundant source of different

genomes. However, within the teleosts, the Ostariophysi (and thus zebrafish) retain many primitive characteristic and occupy a relatively basal position [**276**]. Zebrafish is therefore a rather generalized teleost and can, in most cases, be used to represent the ancestral condition in comparison with more modern laboratory teleosts like medaka and fugu [**276**,**277**] and, with an evolutionary separation of less than 150 million years, the zebrafish is still closer to the aquacultural fish species than any model organism [**278**]. This evolutionary proximity is reflected in many aspects of their biological similarity and offers several advantages for fish nutrition studies. As an example, it has been demonstrated that there is abundant synteny between the zebrafish and genomes salmonids like rainbow trout (*Oncorhynchus mykiss*) and Atlantic salmon (*Salmo salar*) at the level of small regions that include conserved genes [**272**,**279**]. As a consequence, it is possible to use the genomic resources that zebrafish possess and adapt them for use in fish species of economic importance. Furthermore, modern aquaculture, especially intensive industrial aquaculture, involves the production of fish species from captive broodstock, taking care of all aspects of the life cycle including nutrition, welfare, and reproduction. Zebrafish is proposed as a model organism for growth studies due to its metabolic plasticity, with nutritional pathways similar to those of farmed herbivores, such as carp and some tilapias and to those of carnivores such as trout or salmon [**267**,**278**,**280**]. Furthermore, several stressors like handling, hypoxia and confinement or immune system challenging like infection by typical viruses or bacteria commonly present in farmed fish have been applied in zebrafish laboratory trial to mimic fish farm conditions [**252**]. Finally, the reproductive system is well conserved among all fish species, at least with respect to basic molecular and physiological features of the brain-pituitary-gonad axis [**281**]. Thus, zebrafish can be useful to understand the biology of reproduction processes and to extrapolate the results obtained to cultured species, helping to solve aquaculture reproduction-related problems.

Research in the field of fish nutrition and aquaculture must take advantages of and find answers in model organisms like zebrafish where studies in response to alternatives to the conventional ingredients for aquafeeds formulation could potentially be translational to farmed fish species.

Siberian sturgeon

Among 27 sturgeon species in Acipenseridae family, Siberian sturgeon (*Acipenser baerii*; Figure 3) is one of the best species for aquaculture because of fast growing, stress resistance, and adaptation to farming condition [**282**,**283**]. Siberian sturgeon can be reared monoculture in raceways, circular tanks, artificial ponds, recirculation systems, nets, cages, and earthen ponds under temperate conditions [**284**]. Although this is a Eurasian fish and lives in temperate waters, it has been demonstrated that Siberian sturgeon grows well also in high temperatures. Its great interest for

aquaculture sector is due to the production of caviar and high-quality meat for human consumption. Males are reared for meat production (2-3 years), females remain in culture ponds for 6-8 years for caviar production [**285**].

Figure 3. Siberian sturgeon, *Acipenser baerii*. Author: Matteo Zarantoniello.

Siberian sturgeon is typically considered an excellent bottom-feeders because of sensitive barbells underside the long snout to find food and a protruding lip for sucking up the prey [**286**]. They have a unique gastrointestinal tract (Figure 4). Proceeding in caudal direction, the oesophagus, at mid-height, reaches the swim bladder, which is connected by the pneumatic duct junction, and after that starts the stomach that possess a wall hypertrophied as a gizzard-like organ [**287**]. At the end of the swim bladder, the stomach forms a caudal curve, for which we coined the term "large gastric curve", increases in diameter and then it passes to the left side of the visceral cavity. It proceeds in cranial direction, touches the liver, and forms a second curve, named "small gastric curve" [**287**]. The small intestine begins next to the pylori and its right wall is immediately and widely anastomosed to the pyloric caecum, which is located on the left visceral side between the small gastric curve and the small intestine. In caudal direction, the small intestine forms a short tract with two elbow curves, named "S curve", in tight relation to the spleen. The last tract of the gut contains the spiral valve followed by the rectum in the visceral caudal region [**287**].

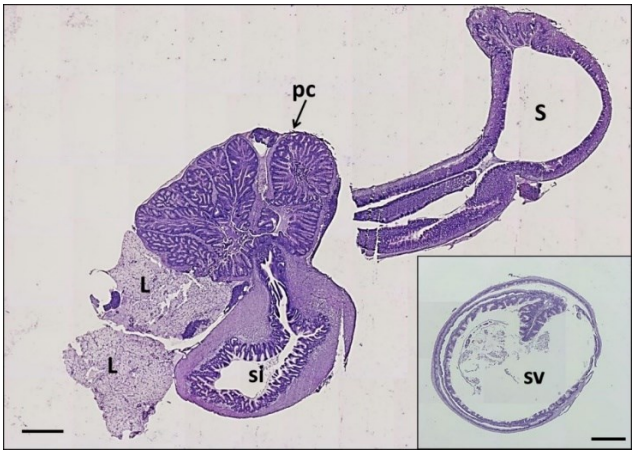

Figure 4. Digestive system histology of Siberian sturgeon (*Acipenser baerii*). Scale bars: main image = 1000 µm; insert = 500 µm. Letters: S = stomach; SI = small intestine; pc = pyloric caecum; L= liver; sv = spiral valve.

In farming condition, Siberian sturgeon can easily adapt to formulated diets based on conventional ingredients (both marine and plant -derived ones). However, after more than four decades of sturgeon rearing, there is a lack of nutrition and feeding information. Therefore, due to an increased interest in Siberian sturgeon aquaculture and restocking programs, it is necessary to find nutritional requirements of these fish and to evaluate physiological responses to alternative dietary ingredients.

Gilthead seabream

The gilthead seabream (*Sparus aurata*; Figure 5) is a subtropical Sparidae distributed naturally in the Mediterranean and the Black Sea (rare), and in the Eastern Atlantic, from the British Isles, Strait of Gibraltar to Cape Verde and around the Canary Islands [**288**]. In the wild, this species inhabits seagrass beds and sandy bottoms as well as the surf zone, commonly to depths of about 30 m, but adults may occur at 150 m depth. It is an euryhaline species and moves, in early spring, towards protected coastal waters in search of more abundant food and warmer temperatures (trophic migration). In late autumn, it returns to the open sea being very sensitive to low temperatures and this migration may coincide with reproduction. The gilthead seabream is a protandrous hermaphrodite: it is a functional male in the first two years and the bisexual gonad has functional testicular, with asynchronous spermatogenesis, and non-functional ovarian areas [**289**]. Over 30 cm in length becomes female, the ovarian development is also asynchronous, and females are batch spawners. In the Mediterranean, the reproductive season ranges between October and December. The eggs are spherical and pelagic, with a diameter slightly lower than 1 mm and a single large oil droplet. Gilthead seabream larvae generally deplete their yolk sacs after 3-4 days of endogenous feeding. At this stage, the eyes are pigmented, and the mouth developed allowing the larvae to prey [**290**].

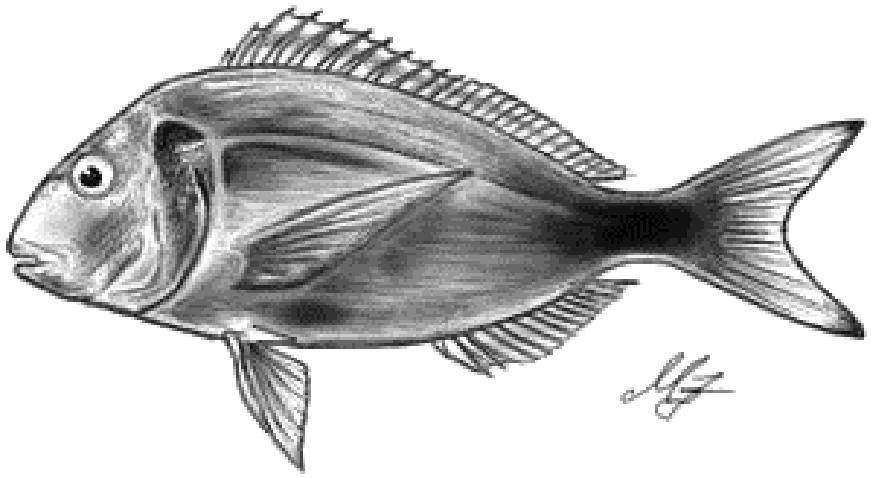

Figure 5. Gilthead seabream, *Sparus aurata*. Author: Matteo Zarantoniello.

Gilthead seabream is mainly carnivorous (shellfish, including mussels and oysters), accessorily herbivorous [**288**]. In the first part of the digestive tract, oesophagus enters a Y-shaped cardiac stomach with a muscular tunica composed of striated fibres in the first part that are gradually replace by smooth ones. The heavily thickened longitudinal muscularis appears on the external wall and persists for all the rest of the digestive tract. The beginning of the intestine is marked by four short pyloric caeca, at the base of which the ductus hepaticus and the ductus pancreaticus discharge. Pyloric caeca have a simple columnar epithelium, with more abundant and longer microvilli than in the intestine. In the intestine, the muscularis and submucosa are very thin in the juvenile but develop in the adult. The intestine mucosa is typically folded and composed of columnar, monolayered enterocytes, with microvilli in their lumen surfaces. Within enterocytes, numerous mucus-secreting cells are present that increase in number in the lower part of the intestine. The intestines forms two bends. After the second bend there is a narrowing corresponding to a valve after which the epithelium shows a different aspect: above the nucleus, cells contain many vacuoles rich with large and eosinophilic granules. The liver is composed by two lobes, not completely divided; the left lobe, much larger, protrudes posteriorly. The ductus hepaticus enters the first part of the intestine, after running parallel to a short ductus pancreaticus [**291**].

Gilthead seabream has a strong economic importance in Mediterranean aquaculture. This fish species can be farmed in coastal ponds and lagoons, with extensive and semi-intensive methods, or in land-based installations and in sea cages, with intensive farming systems [**288**]. These methods are very different, especially regarding fish farming density and food supply. On average, larger pre-fattened gilthead seabream (10 g) reach first commercial size (350-400 g) in about one year, while smaller juveniles (5 g) reach the same size in about 16 months [**288**]. The seabream industry could be described as a sector already entering its mature phase with a well-defined production chain that, however, still rely on the use of large quantities of commercial feeds, mainly based on marine-derived ingredients.

1.9

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Chapter 2

Effects of full-fat BSF-based diets on zebrafish growth, welfare and reproduction

2.1

A six-month study on Black Soldier Fly (*Hermetia illucens*) based diets in zebrafish

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Abstract

Intensive fish farming relies on the use of feeds based on fish meal and oil as optimal ingredients; however, further development of the aquaculture sector needs new, nutritious and sustainable ingredients. According to the concept of circular economy, insects represent good candidates as aquafeed ingredients since they can be cultured through environmental-friendly, cost-effective farming processes, on by-products/wastes, and many studies have recently been published about their inclusion in fish feed. However, information about the physiological effects of insect-based diets over the whole life cycle of fish is presently missing. At this regard, the present study investigated, for the first time, the effects of Black Soldier Fly based diets (25 and 50 % fish meal substitution) administration for a six-month period in zebrafish (*Danio rerio*), from larvae to adults. A multidisciplinary approach, including biometric, biochemical, histological, spectroscopic and molecular analyses was applied. Aside a general reduction in fish growth and lipid steatosis, six-month feeding on Black Soldier Fly based diets did not show major negative effects on zebrafish. Histological analysis on intestine samples did not show signs of inflammation and both stress markers and immune response markers did not show significant differences among the experimental groups.

Introduction

The growing development of a modern aquaculture is strictly connected to a continuous search for sustainable feed ingredients able to promote optimal fish growth and welfare. Aquafeeds have been for a long time based on fish meal (FM) and fish oil (FO) [**1**]. Although these ingredients represent the ideal feed components for fish, they are expensive and often in low supply [**2**]. Consequently, several different alternative ingredients, in particular of plant origin [**3-5**], have been investigated, and some of them are currently used in aquafeed [**6**]. Nevertheless, no one of these alternatives is able to perfectly replace FM or FO due to their inadequate protein and lipid quantity and quality, unbalanced amino acid profile, poor protein digestibility and/or the presence of anti-nutritional factors [**7**]. Recently, insects have received great attention as a new ingredient for aquafeed [**8**] since they show many advantages like a low environmental impact, the ability to grow on waste and by-products, a high feed conversion efficiency and a low risk of transmitting zoonotic infections [**9-11**]. Furthermore, as reported in several reviews on the use of insects in aquafeed [**12**,**13**], insects are characterized by high quantity (60-80%) and quality of protein and are rich in essential amino acids. In particular, Diptera larvae show an essential amino acid composition similar to that of FM [**14**]. However, insects are known to have some critical aspects for fish nutrition such as an unbalanced fatty acid profile [rich in saturated fatty acids (SFA) rather than in polyunsaturated ones (PUFA)] and the presence of chitin [**15**], characteristics that might affect fish growth and welfare, especially when

high inclusion levels are used [**16-18**]. Among approximately one million known insect species, the Black Soldier Fly (*Hermetia illucens*; BSF) is receiving a growing attention in feed formulation. Several studies have been published over the last years about its inclusion in aquafeeds, but results are still controversial and exclusively focused on a short part of fish life cycle. Some studies were performed during the larval/juvenile phase [**11**,**16**,**19**,**20**] while others during the growth-out phase [**9**,**21**,**22**]. Information about the effects of insect-based diets over the whole life cycle of fish is presently missing.

For this reason, the present study investigated, for the first time, the effects of BSF-based diets administration, from larvae to adults in zebrafish (*Danio rerio*). In the present study, emphasis has been given to the adult stage since information about the larval responses to the same diets has recently been published [**11**]. Compared to conventional aquaculture species like trout, turbot, salmon, gilthead seabream and European seabass, zebrafish has the advantage to have a short life cycle (from embryo to adult in about six months) and to provide abundant biological information from genomic sequencing [**23-27**]. The present work represents the first comprehensive multidisciplinary study integrating biometric, histological, molecular, gas chromatographic and spectroscopic analyses, in order to better evaluate the biological and physiological responses to the inclusion of insect meal in aquafeeds during the whole zebrafish life cycle.

Methods

Ethics

All procedures involving animals were conducted in line with the Italian legislation on experimental animals and were approved by the Ethics Committee of the Università Politecnica delle Marche (Ancona, Italy) (84/94-A). All efforts were made to minimize animal suffering by using an anaesthetic (MS222; Merck KGaA, Darmstadt, Germany).

Diets

Three dietary treatments, including increasing levels of full fat Black Soldier Fly (BSF) prepupae meal, were tested in the present study. BSF prepupae were purchased from a commercial company (Smart Bugs s.s. Company, Ponzano Veneto, TV, Italy), where the insects were reared on a substrate composed by corn meal and fruit and vegetable mixture (50:50). Once collected, BSF prepupae were frozen (-80 °C), freeze-dried and minced using liquid nitrogen. The diets were formulated to be iso-nitrogenous and iso-lipidic with full-fat BSF prepupae replacing 25 (Group A) or 50 % (Group B) of the FM/FO of the Control diet, respectively.

All the experimental diets were sieved to obtain a different granulometry as a function of fish size development (as reported in feeding schedule section). Diets (in triplicate subsamples) were analysed for proximate composition and gross energy content measured by an adiabatic bomb calorimeter (IKA C7000, Werke GmbH and Co., Staufen, Germany) [**28**]. For details, please see Table 1.

Table 1. Ingredient composition, proximate analysis and gross energy content of the test diets.

	Control	**Group A**	**Group B**
Ingredients (g/kg)			
Fish meal, Chile, super prime	420	315	210
Peas, protein concentrate	55	78	100
Hermetia illucens meal	0	105	210
Wheat, gluten meal	55	78	100
Wheat flour	290	268	255
Fish oil	70	40	28
Palm oil	70	75	56
Min. & Vit. Supplement [1]	20	20	20
Binder	20	20	20
L-methionine	0	1	1
Proximate composition (%)			
Moisture	4.20±0.03	5.50±0.18	5.30±0.42
Crude protein	40.0±0.5	40.2±0.4	41.1±0.1
Crude lipids	18.6±0.1	17.7±0.2	17.0±0.1
Ash	14.2±0.2	14.1±0.3	12.2±0.6
N-free extractive	23.0±0.3	22.5±0.6	24.4±1.0
Gross Energy (MJ/kg)	22.1±0.1	22.3±0.1	21.3±0.1

[1] Composition of mineral mix (g/kg diet): Ca HPO_4 *$2H_2O$, 27.5; K_2HPO_4, 19.0; NaCl, 6.1; MgO, 2.0; $FeCO_3$, 1.75; KI, 0.15; ZnO, 0.11; MnO, 0.07; $CuSO_4$, 0.02; sodium selenite, 0.002. Composition of vitamin mix (mg/kg diet): thiamine HCl, 40; riboflavin, 40; pyridoxine HCl, 40; cyanocobalamin, 0.2; niacin, 300; calcium pantothenate, 100; folic acid, 5; biotin, 3; choline chloride, 5000; myo-inositol, 1000; ascorbic acid, 2000; a-tocopheryl acetate, 250; menadione, 90; vit. A retinyl palmitate, 40,000 IU/kg diet; vit. D3 cholecalciferol,2500 IU/kg diet.

Fish

Zebrafish (*Danio rerio*) AB embryos (for details about the strains please visit https://zfin.org/ ZDB-GENO-960809-7) were spawned and maintained 48 h in a Tecniplast system (Varese, Italy), subjected to the following conditions: 28 °C, pH 7.0, NH_3 and NO_2^- concentrations < 0.01 mg/L, NO_3^- < 10 mg/L, and photoperiod 12 L/12D. After this first period, embryos were gently collected, counted under a stereomicroscope (Leica Wild M3B, Leica Microsystems, Nussloch, Germany) and randomly divided in three experimental groups (in triplicate) according to the three test diets. In particular, Control group fed fish meal/fish oil diet; Group A fed the diet including 25% full-fat BSF prepupae meal; Group B fed the diet including 50% of full-fat BSF prepupae meal.

Experimental design

Zebrafish larvae were initially reared in 9 tanks (20 L, 3 tanks per experimental group with 500 fish per tank, 1500 per dietary group) and fed the three experimental diets in a tank system according to Olivotto et al. [**29**] and Falcinelli et al. [**30**]. After 30 days post fertilization (dpf), fish of each tank were transferred in bigger tanks (100 L; 9 in total, 3 per each dietary group) equipped with mechanical and biological filtration (Panaque, Rome, Italy) and fed the same diets for 6 months. Six-month-old adult zebrafish were collected and anesthetized with a lethal dose of MS222 (1 g/L, Merck KGaA), counted to estimate survival rate, and the liver, digestive tract and *in toto* fish were sampled and properly stored for further analyses.

Feeding schedule

Starting from 5 dpf to 6 months, fish were fed as follows. Control group: zebrafish fed FM/FO diet; Group A: zebrafish fed the diet including 25% full-fat BSF prepupae meal; Group B: zebrafish fed the diet including 50% of full-fat BSF prepupae meal. Feed particle sizes were < 100 μm from 5 to 15 dpf, 101-200 μm from 16 to 30 dpf, 201-400 μm from 31 to 60 dpf and 401-600 μm from 61 until the end of the experiment. Zebrafish were fed the experimental diets (2% body weight) twice a day [**31**] and, in addition, from 5 to 10 dpf, all groups were fed (one feeding in the morning) the rotifer *Brachionus plicatilis* (5 ind/mL) according to Lawrence et al. [**32**].

Growth and survival

For growth measurements, 20 fish per tank (60 per dietary group) were randomly collected at 6 months and individually measured and weighed. The standard length was determined by a sliding calliper and the weight by an analytical balance (precision: 0.1 mg). Survival was evaluated at the end of the experiment (six months) by counting the number of fish respect to the initial larvae.

Lipid content and fatty acids composition

Experimental diets (3 sample per experimental diet) and whole fish (5 fish per tank, 15 per dietary group) were analysed for lipid content and fatty acids composition. In toto fish were minced, homogenized (homogenizer MZ 4110, DCG Eltronic, Monza, Italy), freeze-dried (Edwards EF4, Crawley, Sussex, England) and lipid extraction was carried out on lyophilized powders following a microwave-assisted extraction [**33**,**34**]. Fatty acids methyl esters (FAME) were prepared according to Truzzi et al. [**34**], using the methyl ester of nonadecanoic acid (19:0; Dr. Ehrenstorfer GmbH, Augsburg, Germany) as internal standard. FAME were determined by an Agilent-6890 GC System (Milano, Italy) coupled to an Agilent-5973N quadrupole Mass Selective Detector (Milano, Italy). A

CPS ANALITICA CC-wax-MS (30 m × 0.25 mm ID, 0.25 μm film thickness) capillary column was used to separate FAME. Instrumental conditions for the studied matrices were set up, according to Truzzi et al. [**34**]. For each sample, at least three runs were performed on the GC-MS. The precision of the proposed method evaluated as in Truzzi et al [**35**] and the limits of detection (LOD) and quantification (LOQ), calculated as in Truzzi et al [**36**], were as in Zarantoniello et al [**11**].

Histology

Intestines and livers collected from 5 different fish specimens for tank (15 per dietary group) were fixed by immersion in Bouin's solution (Merck KGaA) and stored at 4 °C for 24 h. Samples were prepared according to Giorgini et al. [**37**] and 5 μm sections were stained with Mayer hematoxylin and eosin Y (Merck KGaA). Sections were observed using a Zeiss Axio Imager.A2 (Oberkochen, Germany) microscope; images were acquired by mean of a combined colour digital camera Axiocam 503 (Zeiss).

FTIRM analysis

Livers from 5 different fish specimens for tank (15 per dietary group), were quickly dissected and immediately frozen at -80 °C. Then, from the middle part of each sample, three thin sections (10 μm thick) were cut at 100 μm intervals by using a cryomicrotome (Microm HM 505 N, Neuss, Germany) and deposited onto CaF_2 optical windows (1 mm thick, 13 mm diameter) for FTIRM analysis [**37**]. IR measurements were performed at room temperature by using a PerkinElmer Spectrum GXI Spectrometer (Waltham, Massachusetts, USA), equipped with a PerkinElmer AutoIMAGE microscope and a photoconductive HgCdTe MCT array detector, operating at liquid nitrogen temperature (Spectrum Image 5.1.0 software package, Perkin Elmer). By means of a microscope television camera, on each section specific areas were selected on which the IR maps (~560 × 440 μm^2) were acquired in transmission mode in the spectral range 4000 to 800 cm^{-1} with a spectral resolution of 4 cm^{-1}. Background spectra were obtained on clean portions of CaF_2 optical windows. IR maps are false colour images representing the topographical distribution of the total intensity of the infrared absorption within the mapped area; they were made up of 154 pixel/spectra with a spatial resolution of 40 × 40 μm^2. Each IR spectrum was the result of 128 scans. Raw IR maps were corrected for the contributions of carbon dioxide and water vapor and vector normalized on the full frequency range (to avoid artifacts due to local thickness variations).

On each processed IR map, the topographical distribution of lipids, proteins, saturated alkyl chains, phosphate groups and glycogen was obtained by integration under the following spectral regions (OPUS 7.1 software package, Bruker Optics): 3000-2827 cm^{-1} (representative of lipids, LIP); 1700-

1481 cm^{-1} (representative of proteins, PRT); 1481-1429 cm^{-1} (representative of saturated alkyl chains, CH2); 1280-1189 cm^{-1} (representative of phosphate groups, PH), and 1066-975 cm^{-1} (representative of glycogen, GLY). An arbitrary colour scale was used: white colour indicated the pixel with the highest IR absorbance values, while blue colour the lowest ones.

For each map, the absorbance average spectrum, together with its standard deviation spectra (absorbance average spectra ± standard deviation spectra) were calculated (OPUS 7.1 software package, Bruker Optics, Billerica, Massachusetts). Spectra were interpolated in the 3050-2820 cm^{-1}, 1790-1480 cm^{-1} and 1280-1000 cm^{-1} ranges, straight baseline corrected, vector normalized and then curve fitted by using Gaussian curves (Grams A/I 9.1 software package, Galactic) in the same intervals. The position and the area integrals of all the underlying bands were obtained, and these latter used to calculate specific band area ratios (for details, please see Results section).

RNA extraction and cDNA synthesis

Total RNA extraction from both intestine and liver samples from 5 different specimens from each tank (15 fish per dietary group) was performed using RNAzol RT reagent (Merck KGaA) following the manufacturer's protocol. The final RNA concentration was determined by a NanoPhotometer P-Class (Implen, München, Germany) and the RNA integrity was verified by GelRed™ staining of 28S and 18S ribosomal RNA bands on 1% agarose gel. The cDNA synthesis was performed using the high-capacity cDNA reverse transcription kit (Bio-Rad, Hercules, CA, USA) using 3 µg of total RNA.

Real-time PCR

PCRs were performed with SYBER Green in an iQ5 iCycler thermal cycler (both from Bio-Rad) in triplicate. Reactions (10 µL) were run according to Zarantoniello et al. [**11**]. The thermal profile for all reactions was: 3 min at 95 °C, followed by 45 cycles of 20 s at 95 °C, 20 s at the specific annealing temperature of each primer (Table 2) and 20 s at 72 °C. Fluorescence was monitored at the end of each cycle. Melting curve analysis showed a single pick in all cases. Relative quantification of the expression of genes involved in fish growth (insulin-like growth factor 1, *igf1*; insulin-like growth factor 2a, *igf2a;* myostatin, *mstnb*), stress response (glucocorticoid receptor, *nr3c1*; heat shock protein 70, *hsp70.1*), long-chain polyunsaturated fatty acids biosynthesis (fatty acid elongase 2, *elovl2*; fatty acid elongase 5, *elovl5;* fatty acid desaturase 2, *fads2*), immune response (interleukin 1β, *il1b*; interleukin 6, *il6*; tumor necrosis factor a, *tnfa*) and enzymatic hydrolysis of chitin (chitinase 2, *chia.2*; chitinase 3, *chia.3*; chitinase 5, *chia.5*) was performed. Actin-related protein 2/3 complex, subunit 1A (*arpc1a*) and ribosomal protein, large, 13 (*rpl13*) were used as internal standards in each sample to standardize the results by eliminating variation in mRNA and cDNA quantity and quality.

Amplification products were sequenced, and homology was verified. No amplification product was detected in negative controls and no primer-dimer formation was found in control templates. Data were analysed using the iQ5 optical system software version 2.0, including Genex Macro iQ5 Conversion and Genex Macro iQ5 files (all from Bio-Rad). Modification of gene expression was reported with respect to controls. Primer sequences were designed using Primer3 (210 v. 0.4.0) starting from zebrafish sequences available in ZFIN (for details, please see Table 2).

Table 2. Sequences, Zebrafish Information Network (ZFIN) IDs, and annealing temperature (A.T.) of the primers used for the present study.

Gene	Forward Primer (5'-3')	Reverse Primer (5'-3')	ZFIN IDs	A. T. (°C)
igf1	GGCAAATCTCCACGATCTCTAC	CGGTTTCTCTTGTCTCTCTCAG	ZDB-GENE-010607-2	53
igf2a	GAGTCCCATCCATTCTGTTG	GTGGATTGGGGTTTGATGTG	ZDB-GENE-991111-3	59
mstnb	GGACTGGACTGCGATGAG	GATGGGTGTGGGGATACTTC	ZDB-GENE-990415-165	58
nr3c1	AGACCTTGGTCCCCTTCACT	CGCCTTTAATCATGGGAGAA	ZDB-GENE-050522-503	58
hsp70.1	TGTTCAGTTCTCTGCCGTTG	AAAGCACTGAGGGACGCTAA	ZDB-GENE-990415-91	58
elovl2	CACTGGACGAAGTTGGTGAA	GTTGAGGACACACCACCAGA	ZDB-GENE-011212-1	60
elovl5	TGGATGGGACCGAAATACAT	GTCTCCTCCACTGTGGGTGT	ZDB-GENE-040407-2	60
fads2	CATCACGCTAAACCCAACA	GGGAGGACCAATGAAGAAGA	ZDB-GENE-011212-1	60
il1b	GCTGGGGATGTGGACTTC	GTGGATTGGGGTTTGATGTG	ZDB-GENE-040702-2	54
il6	CTGGAGGCCATAAACAGCCA'	TGCGAGTCCATGCGGATTTA'	ZDB-GENE-120509-1	58
tnfa	TTGTGGTGGGGTTTGATG	TTGGGGCATTTTATTTTGTAAG	ZDB-GENE-050317-1	53
chia.2	GGTGCTCTGCCACCTTGCCTT	GGCATGGTTGATCATGGCGAAAGC	ZDB-GENE-040426-2014	64
chia.3	TCGACCCTTACCTTTGCACACACCT	ACACCATGATGGAGAACTGTGCCGA	ZDB-GENE-040426-2891	65
chia.5	CCACGGCTCACAGGACAACATCA'	GTCCGCAGACGACAGGCGAA'	ZDB-GENE-071004-113	60
arpc1a	CTGAACATCTCGCCCTTCTC	TAGCCGATCTGCAGACACAC	ZDB-GENE-040116-1	60
rpl13	TCTGGAGGACTGTAAGAGGTATGC	AGACGCACAATCTTGAGAGCAG	ZDB-GENE-031007-1	59

Statistical analysis

All data were analysed by one-way ANOVA, with diet as the explanatory variable. All ANOVA tests were followed by Tukey's post-hoc test. The statistical software package Prism 6 (GraphPad Software) was used. Significance was set at $p < 0.05$ and all the results are presented as mean ± SD.

Results

Growth and survival

As concerns standard length, both Group A (27.6 ± 2.0 mm) and Group B (26.4 ± 1.6 mm) were significantly smaller than Control (29.8 ± 1.9 mm). Considering wet weight, Group B (0.21 ± 0.04 g) showed a significant lower value than Control (0.28 ± 0.03 g), while no significant differences were observed between Control and Group A (0.25 ± 0.04 g). Considering survival at six months, no significant differences were observed among the experimental groups. Group A reached the highest

survival value (65 ± 11%), while Control and Group B showed a 60 ± 9 and 58 ± 7 % survival, respectively.

Fatty acids content and composition

Diets. The fatty acids content (as % of total fatty acids) of the three experimental diets is presented in Figure 1a. The increasing inclusion levels of BSF full-fat prepupae meal in the diets resulted in a statistically significant increase of SFA content (33.8 ± 0.4, 42.1 ± 4.5 and 48.2 ± 1.2 % for Control, A and B diets, respectively) and a parallel significant decrease in PUFA content (33.0 ± 1.4, 23.0 ± 1.1 and 18.5 ± 2.1 % for Control, A and B diets, respectively). In particular, as concern both n3 and n6 contents, Control diet (22.0 ± 0.4 and 11.3 ± 2.2 %, respectively) showed a significantly higher percentage with respect to diet A (13.7 ± 0.2 and 9.7 ± 1.6 %, respectively) and diet B (10.1 ± 0.3 and 8.6 ± 1.5 %, respectively). Differently, considering monounsaturated fatty acids (MUFA) first and n9 content secondly, no significant differences were detected between Control (32.9 ± 1.2 and 26.5 ± 0.7 %, respectively) and B diets (33.0 ± 1.4 and 25.5 ± 2.1 %, respectively), while diet A (34.5 ± 0.6 and 28.0 ± 1.4 %, respectively) was characterized by a significantly higher content with respect to the other diets. Finally, no significant differences were observed for n6/n3 ratio (Fig. 1b) between Control diet (0.5 ± 0.1) and A diet (0.7 ± 0.1), while B diet (0.8 ± 0.1) showed a significantly higher value than Control group.

As regards specific fatty acids composition (Table 3), the most relevant SFA in all the experimental diets was the palmitic acid (16:0), while lauric acid (12:0) increased significantly according to the dietary BSF meal inclusion. Oleic acid (18:1n9) was the most abundant MUFA in all the dietary treatments. Finally, Control diet showed the highest amount of DHA (22:6n3; 22.2 ± 0.9 %) and EPA (20:5n3; 11.3 ± 0.2 %) which significantly decreased in diet A (8.2 ± 0.4 and 4.2 ± 0.5 %, respectively) and B (6.0 ± 0.7 and 3.1 ± 0.7 %, respectively).

Zebrafish. Figure 1c reports the fatty acids content (as % of total fatty acids) of adult zebrafish fed the different diets. The SFA content showed statistically significant differences among experimental groups, increasing from Control (24.0 ± 0.3%) to Group A (27.1 ± 0.3 %) and to Group B (34.4 ± 0.4 %). Regarding MUFA and n9 contents, both Group A (41.9 ± 0.4 and 33.8 ± 0.4 %, respectively) and B (41.7 ± 0.5 and 33.6 ± 0.4 %, respectively) showed a significantly lower percentage respect to Control zebrafish (45.2 ± 0.3 and 37.6 ± 0.3 %, respectively). However, no significant differences were detected between Group A and B. Group B zebrafish showed a significantly lower PUFA percentage (23.9 ± 0.3 %) both respect to Control (30.9 ± 0.5 %) and Group A (31.0 ± 0.5). Considering n6 percentage, no statistically significant differences were observed between Control (15.7 ± 0.2 %) and Group B (15.5 ± 0.2 %), while Group A zebrafish (17.5 ± 0.2 %) showed a

significantly higher percentage than the other groups. Finally, the increasing inclusion levels of BSF full-fat prepupae meal resulted in a statistically significant decrease in n3 percentage (15.2 ± 0.5, 13.5 ± 0.5 and 8.4 ± 0.3 % for Control, Group A and Group B, respectively) and in a statistically significant increase in n6/n3 ratio (1.0 ± 0.1, 1.3 ± 0.1 and 1.8 ± 0.1 for Control, Group A and Group B, respectively; Fig. 1d).

As concern the fatty acids composition of adult zebrafish (Table 3), the most represented SFA in all the experimental groups were palmitic acid (16:0) and stearic acid (18:0). Furthermore, the content of lauric (12:0) and myristic (14:0) acids significantly increased according to the increasing dietary BSF meal inclusion. Considering MUFA, the predominant fatty acid in all the experimental groups was oleic acid (18:1n9) which did not show statistically significant differences among the experimental groups. Linoleic acid (18:2n6) was the most abundant PUFA in all the dietary treatments. In addition, Control group was characterized by a significantly higher percentage of EPA (20:5n3; 3.2 ± 0.5 %) with respect to Group A (2.2 ± 0.3 %) and Group B (1.2 ± 0.1 %). On the other hand, Group B (5.1 ± 0.1 %) showed a significantly lower value of DHA (22:6n3) respect to both Group A (8.5 ± 0.6 %) and Control (8.6 ± 0.2 %).

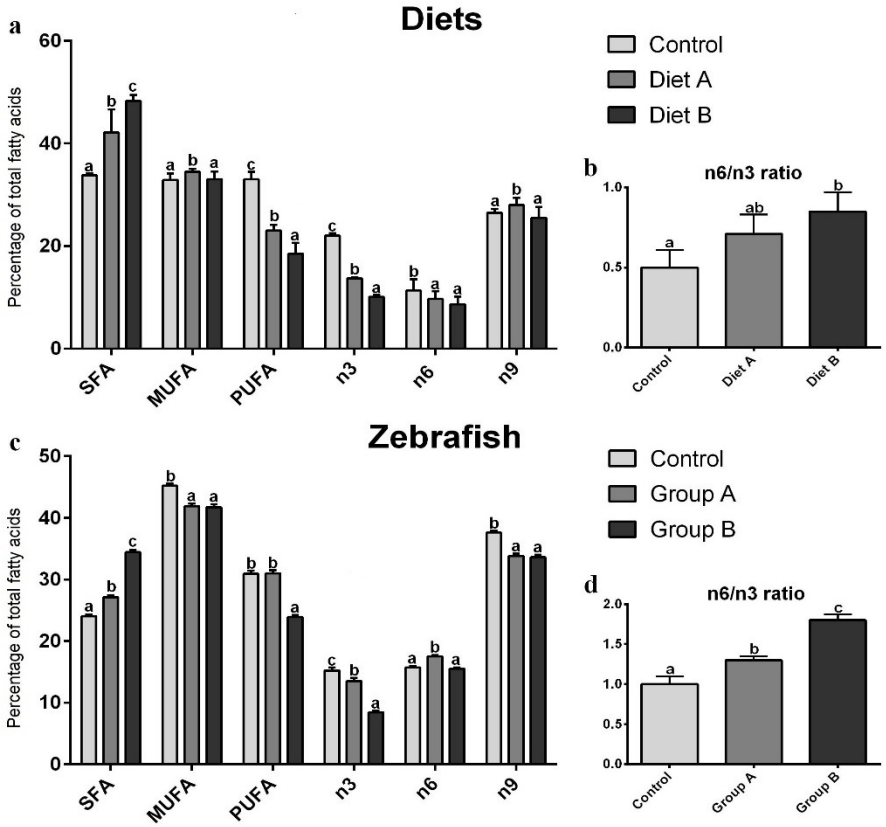

Figure 1. Fatty acids content (as % of total fatty acids) and n6/n3 ratio. (**a**,**b**) experimental diets; (**c**,**d**) adult zebrafish. Control diet was based on FM, while A and B diets were characterized by 25 or 50 % replacement of FM with full-fat BSF prepupae meal, respectively. Different letters indicate statistically significant differences among experimental groups compared within the same fatty acid class ($p < 0.05$). Values are presented as mean ± SD (n = 3 for diets; n = 15 for zebrafish).

Table 3. Fatty acids composition (% fatty acids methyl esters) of experimental diets and adult zebrafish.

	Diets			Zebrafish		
	Control	**Group A**	**Group B**	**Control**	**Group A**	**Group B**
10:0	n.d.	0.4±0.1 [a]	0.7±0.1 [a]	n.d.	0.05±0.01 [b]	0.07±0.01 [c]
12:0	0.1±0.1 [a]	6.5±0.7 [b]	13.0±1.4 [c]	0.3±0.1 [a]	5.5±0.4 [b]	7.7±0.2 [c]
13:0	0.05±0.02 [a]	0.04±0.05 [a]	0.07±0.04 [a]	0.02±0.01 [a]	0.02±0.01 [a]	0.03±0.01 [b]
14:0	4.3±0.2 [a]	4.0±0.3 [a]	5.7±1.0 [b]	2.0±0.1 [a]	3.4±0.2 [b]	4.7±0.5 [c]
14:1n5	n.d.	n.d.	n.d.	0.2±0.1 [a]	0.5±0.1 [b]	1.0±0.1 [c]
15:0	0.7±0.1 [a]	0.4±0.1 [a]	0.5±0.3 [a]	0.4±0.1 [a]	0.4±0.1 [a]	0.4±0.1 [a]
15:1n5	n.d.	n.d.	n.d.	0.2±0.1 [a]	0.2±0.1 [a]	0.3±0.1 [b]
16:0	15.0±1.6 [b]	24.1±2.8 [a]	21.6±1.2 [a]	17.1±0.4 [a]	14.7±0.5 [b]	17.7±0.4 [a]
16:1n9	0.2±0.1 [a]	0.1±0.1 [a]	0.2±0.1 [a]	1.2±0.1 [a]	1.4±0.1 [a]	1.4±0.2 [a]
16:1n7	5.3±0.4 [a]	3.9±0.9 [b]	5.2±0.8 [a]	4.0±0.2 [a]	4.4±0.2 [a]	4.3±0.2 [a]
17:0	0.8±0.1 [a]	0.5±0.1 [a]	0.6±0.1 [a]	0.5±0.1 [a]	0.4±0.1 [a]	0.4±0.1 [a]
17:1n7	n.d.	n.d.	n.d.	0.5±0.1 [a]	0.5±0.1 [a]	0.5±0.1 [a]
18:0	4.9±0.7 [a]	5.4±1.1 [a]	5.1±0.2 [a]	3.5±0.2 [a]	2.4±0.1 [b]	3.2±0.3 [a]
18:1n9	13.2±0.5 [a]	27.8±1.6 [b]	25.00±1.8 [b]	33.6±1.7 [a]	30.4±1.3 [a]	30.7±1.1 [a]
18:1n7	2.1±0.4 [a]	1.0±0.2 [b]	1.0±0.3 [b]	2.8±0.1 [a]	2.4±0.2 [b]	2.0±0.1 [c]
18:2n6	11.2±1.5 [a]	8.9±1.5 [ab]	8.0±1.5 [b]	12.6±0.7 [a]	14.3±0.4 [b]	12.2±0.1 [a]
18:3n6	0.2±0.03 [a]	0.07±0.04 [ab]	0.05±0.06 [b]	0.3±0.1 [a]	0.3±0.1 [a]	0.3±0.1 [a]
18:3n3	2.4±0.1 [a]	1.1±0.2 [b]	0.9±0.3 [b]	3.0±0.1 [a]	2.6±0.3 [a]	1.9±0.3 [b]
20:0	0.4±0.1 [a]	0.4±0.1 [a]	0.4±0.1 [a]	0.1±0.1 [a]	0.1±0.1 [a]	0.1±0.1 [a]
20:1n9	1.4±0.3 [a]	0.9±0.2 [a]	0.9±0.2 [a]	2.0±0.1 [a]	1.4±0.1 [b]	1.1±0.1 [c]
20:2n6	0.9±0.1 [a]	0.1±0.1 [b]	0.1±0.1 [b]	0.9±0.1 [a]	0.7±0.1 [a]	0.7±0.1 [a]
20:3n6	0.20±0.04 [a]	0.07±0.08 [b]	0.05±0.03 [b]	0.8±0.1 [a]	0.9±0.1 [a]	1.0±0.2 [a]
21:0	0.07±0.05 [a]	0.03±0.03 [a]	0.02±0.02 [a]	0.01±0.01 [a]	0.02±0.01 [a]	0.01±0.01 [a]
20:4n6	1.2±0.1 [b]	0.5±0.1 [a]	0.4±0.1 [a]	1.2±0.2 [a]	1.4±0.1 [a]	1.3±0.4 [a]
20:3n3	0.2±0.1 [a]	0.1±0.1 [a]	0.1±0.1 [a]	0.4±0.1 [a]	0.2±0.1 [b]	0.2±0.1 [b]
20:5n3	11.3± 0.2 [a]	4.2±0.5 [b]	3.1±0.7 [b]	3.2±0.5 [a]	2.2±0.3 [b]	1.2±0.1 [c]
22:0	0.3±0.1 [a]	0.3±0.1 [a]	0.4±0.1 [a]	0.08±0.01 [a]	0.08±0.01 [a]	0.05±0.01 [b]
22:1n9	0.9±0.1 [a]	0.4±0.1 [b]	0.3±0.1 [b]	0.7±0.1 [a]	0.5±0.1 [ab]	0.3±0.1 [b]
24:0	0.01±0.01 [a]	0.05±0.02 [a]	0.04±0.05 [a]	n.d.	n.d.	n.d.
22:6n3	22.2±0.9 [c]	8.2±0.4 [b]	6.0±0.7 [a]	8.6±0.2 [a]	8.5±0.6 [a]	5.1±0.1 [b]
24:1n9	0.8±0.4 [a]	0.4±0.1 [a]	0.4±0.1 [a]	0.2±0.1 [a]	0.1±0.1 [a]	0.1±0.1 [a]

For each matrix, mean within rows bearing different letters are significantly different ($p < 0.05$; n = 3 for diets; n = 15 for zebrafish). **Diets**: Control diet was based on FM, while A and B diets were characterized by 25 or 50% replacement of FM with full-fat BSF prepupae meal, respectively. **Zebrafish**: fish fed diet based on FM (Control) and diets with 25 (Group A) or 50 % (Group B) replacement of FM with full-fat BSF prepupae meal.

Histological analyses

Histological analyses were performed on intestine and liver samples and results varied among the experimental groups. All fish, regardless of the diet, did not show any morphological alterations of the intestine (Fig. 2a-c). As concern liver (Fig. 2d-f), results evidenced a variable degree of lipid accumulation in the experimental groups. A moderate intracytoplasmic lipid accumulation was observed in liver from Control group, characterized by a diffuse presence of hepatocytes with

cytoplasm filled of fat, interspersed with normal hepatocytes (Fig. 2d). All liver samples from Group A (Fig. 2e) and, in particular, Group B (Fig. 2f) showed moderate degree of steatosis with swollen hepatocytes and abundant intracytoplasmic lipid accumulation.

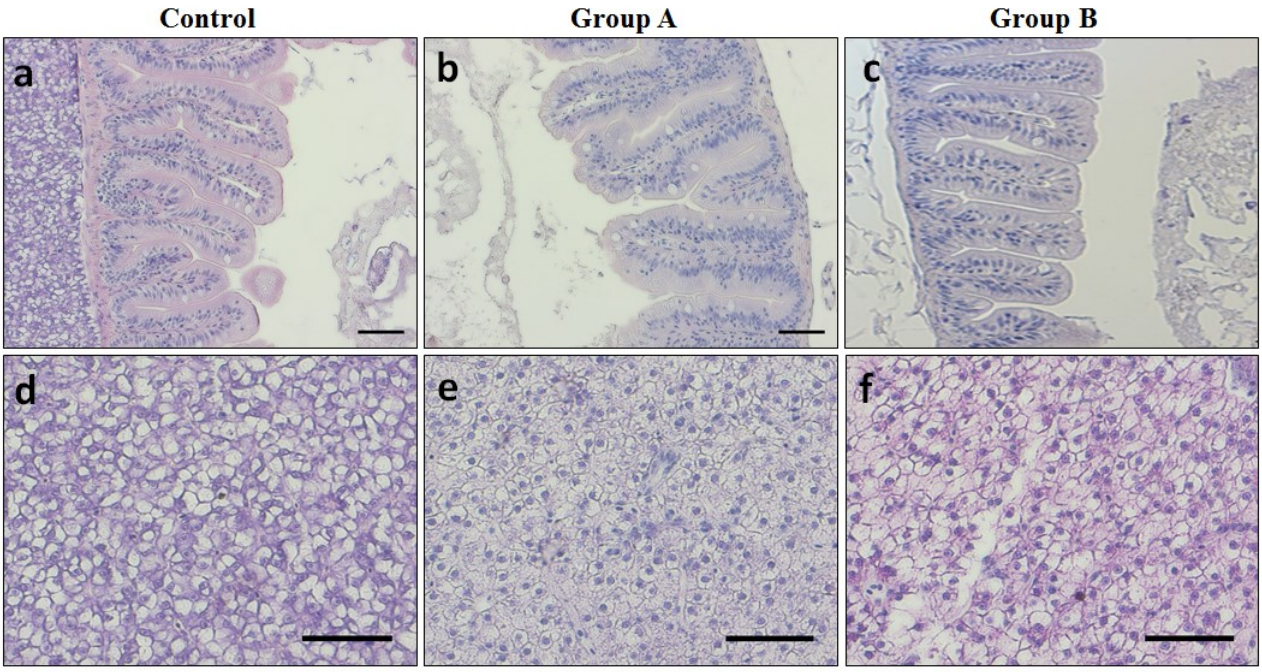

Figure 2. Example of histomorphology of adult zebrafish. (**a-c**) intestine; (**d-f**) liver. Scale bars: (**a-c**) 50 µm; (**d-f**) 100 µm. Zebrafish fed diet based on FM (Control) and diets with 25 (Group A) or 50 % (Group B) replacement of FM with full-fat BSF prepupae meal.

Fourier transform-infrared micro-spectroscopy (FTIRM) analysis

The imaging vibrational analysis of three sections representative of Control, Group A and Group B zebrafish liver samples is reported in Figure 3. With respect to Control liver samples, major amounts of lipids (LIP), saturated alkyl chains (CH2), proteins (PRT), glycogen (GLY) and phosphate groups (PH) were observed in Group B, while an intermediate result was detected in Group A samples.

Due to the complexity of the spectral profile and the presence of several convoluted bands, a semiquantitative analysis of the spectral data was performed. At this purpose, for each experimental group, the absorbance average spectrum, together with its standard deviation spectra (absorbance average spectra ± standard deviation spectra) were calculated and curve fitted in the following spectral ranges: 3050-2820 cm^{-1} (containing the vibrational modes of CH, CH2 and CH3 groups of lipid alkyl chains, and hence representative of lipids), 1790-1480 cm^{-1} (containing the vibrational modes related to proteins secondary structure, and hence representative of proteins), and 1280-1000 cm^{-1} (containing the vibrational modes of carbohydrates and phosphates, and hence representative of glycogen and phospholipids). The integrated areas of specific underlying bands with biological meaning were used to calculate the following band area ratios (Fig. 4): SAT/LIP (relative amount of

saturated alkyl chains in lipids); UNSAT/LIP (relative amount of unsaturated alkyl chains in lipids); CH2/CH3 (degree of saturation and length of lipid alkyl chains); CH/CH3 (degree of unsaturation of lipid alkyl chains); FOLDED/PRT (relative amount of folded structures in proteins); UNFOLDED/PRT (relative amount of unfolded structures in proteins); FA/PRT (relative amount of fatty acids compared to proteins); PH/GLY (relative amount of phosphate groups compared to glycogen), and PHLIP/GLY (relative amount of phospholipids compared to glycogen). By comparing the numerical variation of the above cited band ratios for Control, Group A and Group B liver samples, the following observations can be made: (i) a higher amount of saturated lipid alkyl chains (SAT/LIP and CH2/CH3; Fig. 4a,c) and a lower quantity of unsaturated ones (UNSAT/LIP and CH/CH3; Fig. 4b,d) were observed in Group A and Group B liver samples with respect to Control one; (ii) no statistically significant variation was detected in the relative amount of fatty acids, with respect to proteins (FA/PRT; Fig. 4e) by comparing all the experimental groups; (iii) regards proteins, a decrease of folded structures (FOLDED/PRT; Fig. 4f) and an increase of unfolded ones (UNFOLDED/PRT; Fig. 4g) was detected (even if both not statistically meaningful) in Group A and Group B liver samples; (iv) a higher amount of phosphate groups (PH/GLY; Fig. 4h) and phospholipids (PHLIP/GLY; Fig. 4i) with respect to glycogen was noticed.

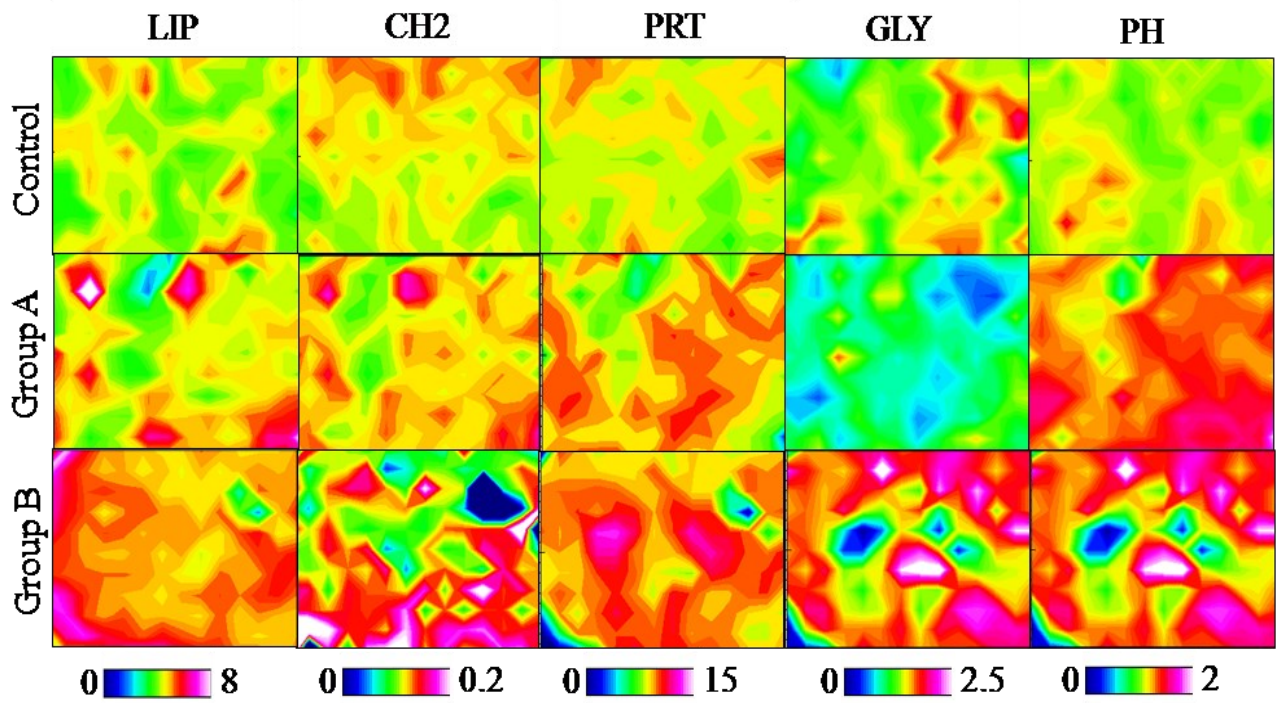

Figure 3. Example of imaging vibrational analysis of Control, Group A and Group B zebrafish liver samples. Topographical distribution of: lipids (LIP); saturated alkyl chains (CH2); proteins (PRT); glycogen (GLY) and phosphate groups (PH). Map size ~560 × 440 μm^2. Zebrafish fed diet based on FM (Control) and diets with 25 (Group A) or 50 % (Group B) replacement of FM with full-fat BSF prepupae meal.

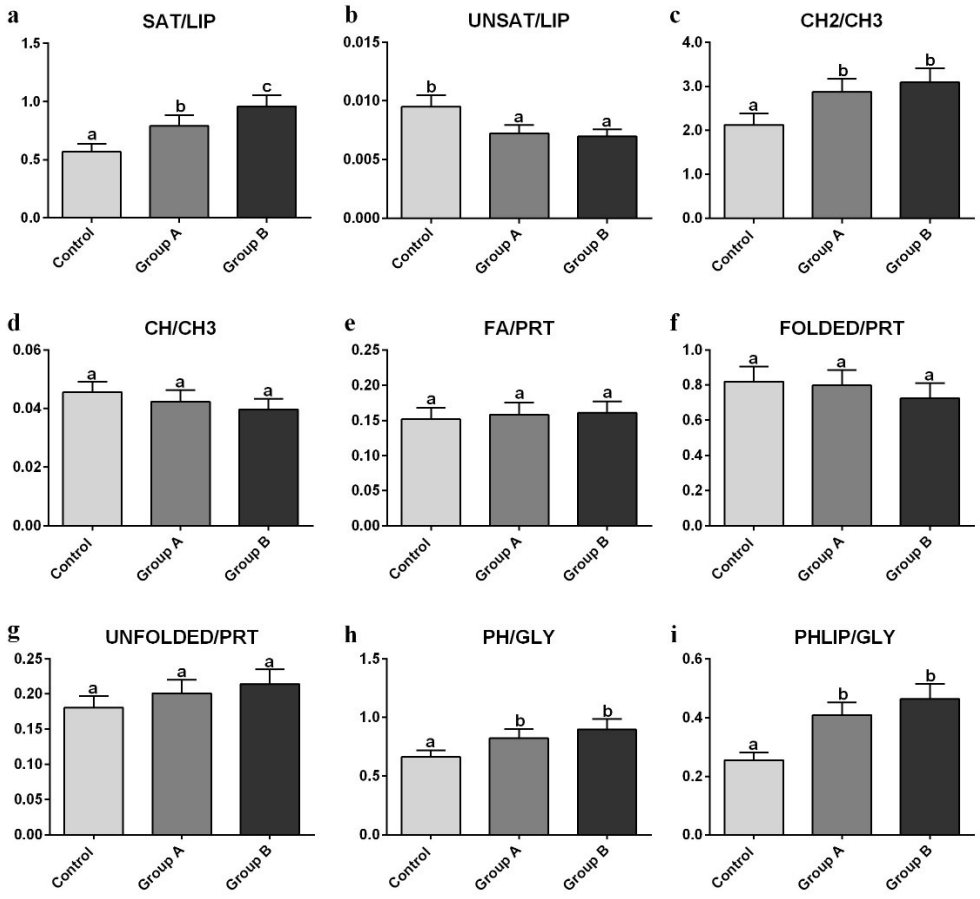

Figure 4. Semiquantitative analysis of the biochemical composition of Control, Group A and Group B liver samples. Statistical analysis of the numerical variation of the following band area ratios: (**a**) SAT/LIP; (**b**) UNSAT/LIP; (**c**) CH2/CH3; (**d**) CH/CH3; (**e**) FA/PRT; (**f**) FOLDED/PRT; (**g**) UNFOLDED/PRT; (**h**) PH/GLY, and (**i**) PHLIP/GLY. Different letters indicate statistically significant differences among experimental groups ($p < 0.05$). Values are presented as mean ± SD (n = 15). Zebrafish fed diet based on FM (Control) and zebrafish fed diets with 25 (Group A) or 50 % (Group B) replacement of FM with full-fat BSF prepupae meal.

Real-time PCR results

Real-time PCR analyses were performed on liver samples in order to test the expression of genes involved in fish growth (*igf1*, *igf2a* and *mstnb*), stress response (*nr3c1* and *hsp70.1*) and long-chain polyunsaturated fatty acids biosynthesis (*elovl2, elovl5* and *fads2*). Differently, genes involved in immune response (*il1b, il6* and *tnfa*) and enzymatic hydrolysis of chitin (*chia.2*, *chia.3* and *chia.5*) were investigated in intestine samples.

Growth factors. Considering both *igfs* gene expression (Fig. 5a,b), no significant differences were detected between Control and Group A, while Group B showed significantly higher values with respect to both Control and Group A. Differently, regarding *mstnb* gene expression (Fig. 5c), no significant differences were observed among all the experimental groups.

Stress response. As concerns both *nr3c1* and *hsp70.1* gene expression (Fig. 5d,e), no significant differences were evident among the experimental groups.

Lipid metabolism. The expression of genes involved in long-chain polyunsaturated fatty acid elongation (*elovl2* and *elovl5*) and desaturation (*fads2*) evidenced a similar pattern (Fig. 5f-h). In particular, the higher the BSF meal inclusion level the higher was the *elovl2*, *elovl5* and *fads2* gene expression. No significant differences were detected between Control and Group A.

Immune response. Considering *il1b* and *tnfa* gene expression (Fig. 5i,m), no significant differences were observed among the experimental groups, while, as concerns *il6* gene expression (Fig. 5l), both Control and Group A showed a significantly higher gene expression compared to Group B.

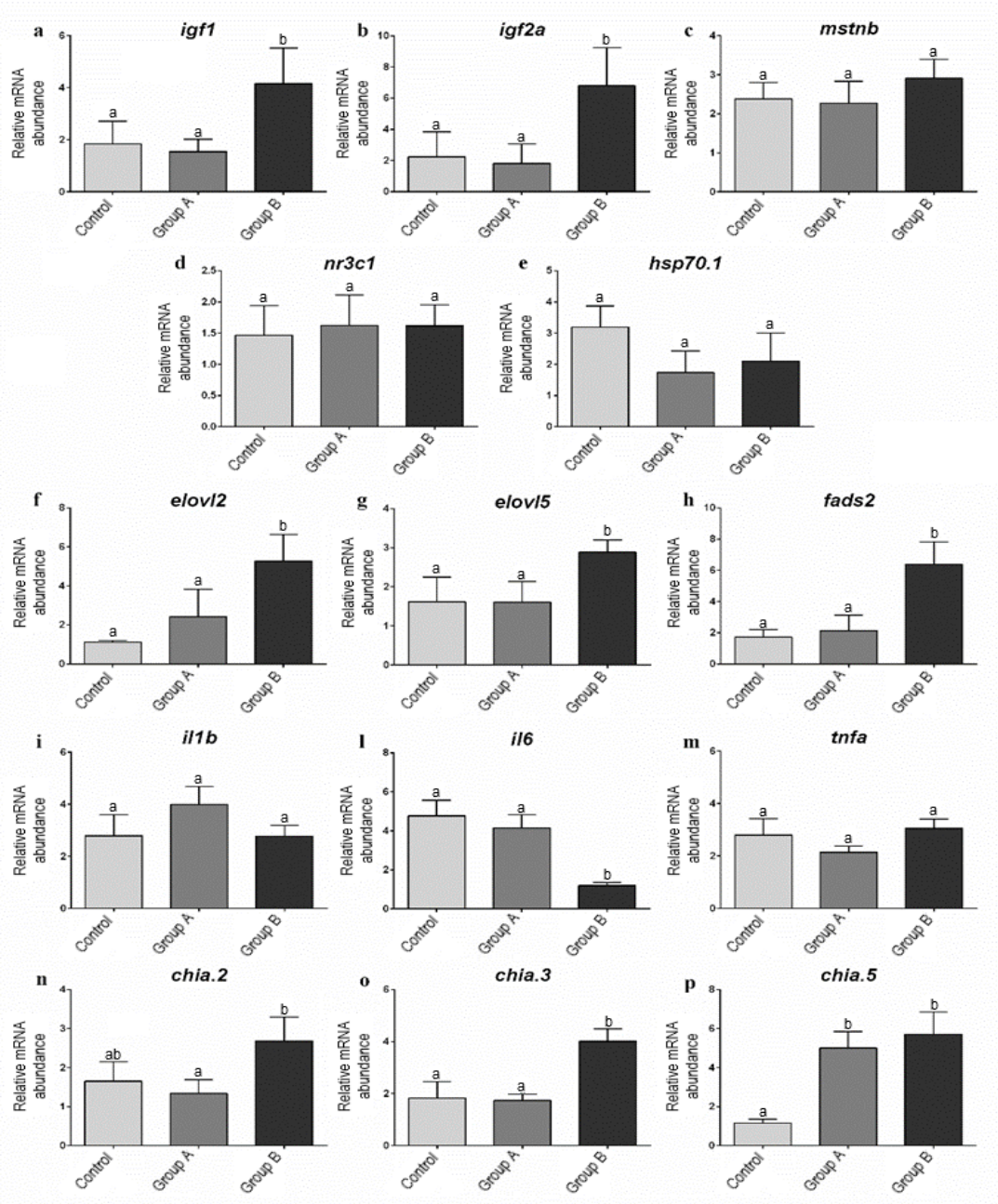

Figure 5. Relative mRNA levels of genes analysed in adult zebrafish. (**a-c**) growth; (**d**,**e**) stress response; **(f-h**) lipid metabolism and long-chain PUFA biosynthesis; (**i-m**) immune response; (**n-p**) enzymatic hydrolysis of chitin. Different letters indicate statistically significant differences among experimental groups ($p < 0.05$). Values are presented as mean ± SD (n = 5). Zebrafish fed diet based on FM (Control) and diets with 25 (Group A) or 50 % (Group B) replacement of FM with full-fat BSF prepupae meal.

Discussion

With the growing understanding of the role of bio-economy within Europe and the need to apply circularity in the use of natural resources, recognizing that the waste can represent a new starting material for other industrial processes is now a must. In this sense, as suggested by the EU community, a circular economy should be applied also to the aquaculture sector, improving economic benefits, contributing to innovation and growth, encouraging sustainability and competitiveness in the long term.

According to the concept of circular economy, insects represent good candidates as aquafeed ingredients, since they can be cultured through environmental-friendly, cost-effective farming processes, on by-products/wastes [**38**,**39**]. In particular, *Hermetia illucens* is one of the most promising insect species especially because of its essential amino acid pattern, similar to that of FM [**40-42**]. The inclusion of BSF meal in aquafeed has been extensively investigated in the last years but results are still controversial. In addition, all the studies so far performed are focused on a short period of fish life cycle (larval stage, juvenile stage or growth-out stage) [**16**,**21**,**43-46**]. To the best of our knowledge, no study about the effects of insect-based diets during the whole life cycle of fish is presently available. One of the possible reasons of this lack of information is mainly related to the fact that most aquaculture species have long life cycles, making these feeding trials expensive and laborious.

At this regard, the use of species that are amenable to experimental investigation provides tools to approach questions that would not be feasible in other "non-model" organisms. For example, small teleost fish such as zebrafish and medaka have the same or similar developmental mechanisms, morphological features and physiological responses of many aquaculture species [**47**] providing the ability to use comparative analyses between different organisms to understand mechanisms of development, physiology and possibly providing useful information for finfish production. For these reasons, the present study represents the first report about a six-months feeding trial based on BSF diets on an experimental model, zebrafish.

As reported in previous studies, insect meal could reduce fish growth and welfare especially over longer periods of time and when high percentages of inclusion (more than 25-30%) are used [**16**,**48**,**49**]. At this regard, the present six months experiment evidenced that, even if the tested diets were iso-energetics, fish growth was negatively affected by the increasing percentage of BSF prepupae meal in the diets. However, biometric results were not fully supported by the molecular ones that evidenced a significantly higher growth factors (*igf1* and *igf2a*) gene expression in fish fed BSF-based diets. Results are not obvious especially because of the pleiotropic nature of this hormones family which is involved in fish growth regulation but also in many other biological processes like

DNA synthesis, cartilage sulfation and protein synthesis, spermatogenesis and final oocyte maturation [**50**]. Fish growth delay is often related to starvation or malnutrition (for example a long-term deficiency of essential nutrients or presence of anti-nutritional factors) [**51**,**52**]. In the present study the delay in growth observed in fish fed BSF-based diets respect to control is probably due to an unbalanced fatty acid composition of the diets. In fact, higher BSF prepupae meal inclusion levels in the diets, resulted in lower HUFA and higher SFA content in the diets.

Many freshwater species, including zebrafish, are able to convert shorter-chain precursors in highly unsaturated fatty acids through the activation of specific elongase and desaturase [**51**,**53**]. After a 6-month treatment period, the fish fed the highest percentage of BSF inclusion showed higher elongase and desaturase (*elovl2*, *elovl5* and *fads2*) gene expression compared to Control, and GC analyses revealed the presence of HUFA, underlying the ability of the fish to promote the above mentioned conversions. These biochemical conversions require energy-expenditure by the fish that may thus explain the observed growth delay. Additionally, zebrafish fed BSF-based diets showed a higher accumulation in SFA respect to Control, particularly evident at the hepatic level. The FTIRM analysis on liver samples evidenced an increase of band area ratios related to SFA in fish fed BSF-based diets. In addition, as already evidenced by previous studies, high SFA, together with a high n6/n3 ratio, caused hepatic steatosis in zebrafish specimens fed BSF-based diets [**54-56**].

Aside a general reduction in fish growth, six months feeding on BSF-based diets did not show major negative effects on zebrafish. Gut histological analysis on intestine samples did not show signs of inflammation. Furthermore, both stress markers (*nr3c1* and *hsp70.1*) and two of the immune response markers analysed (*il1b* and *tnfa*) did not show significant differences among the experimental groups. These results may both be supported by the fact that zebrafish possess specific chitinases able to digest chitin (with a gene expression dependent on BSF meal inclusion level) or by the anti-inflammatory, antibacterial and antiviral properties of medium-chain fatty acids (especially lauric acid, 12:0) which are particularly abundant in the BSF-based diets used in this study [**57-59**].

In conclusion, this is the first report about a six-month feeding study on BSF-based diets during the whole life cycle of a teleost fish (from larvae to adults). Results are encouraging, however, for a wider possible application of insect meal in aquaculture, further research is necessary to improve the insects' fatty acid composition in order to better meet fish nutritional requirements.

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2.2

Can insect-based diets affect zebrafish (*Danio rerio*) reproduction? A multidisciplinary study

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Abstract

Black Soldier Fly (*Hermetia illucens*, BSF) meal is considered an alternative, emerging, and sustainable ingredient for aquafeed formulation. However, results on fish physiological responses are still fragmentary and often controversial, and no data are available on the effect of insect meal-based diets on fish reproduction. On this regard, zebrafish, with its relatively short life cycle, represents an ideal experimental model to explore this topic. In this study, female zebrafish were fed for 12 months a control diet based on fish meal (FM) and fish oil and two experimental diets with full-fat BSF prepupae meal inclusion, to replace 25 and 50 % of FM (BSF25 and BSF50). All diets were isonitrogenous, isolipidic, and isoenergetic. The effects of these two experimental diets on reproduction in female specimens were investigated through a multidisciplinary approach, including the evaluation of growth, gonadosomatic index, spawned/fertilized eggs and hatching rate, fatty acids composition of adult females' carcasses and fertilized egg, histological analysis of the ovary, spectroscopic macromolecular composition of class IV oocytes, and expression of genes involved in fish lipid metabolism in the liver. Results showed that, while fish were perfectly able to cope with a 25% insect meal dietary inclusion, a 50% inclusion level caused the overexpression of genes involved in lipid metabolism, a general reduction in the number of spawned eggs, and differences in terms of: (i) previtellogenic, class III, class IV, and atretic oocytes and postovulatory follicles frequency rate; (ii) macromolecular composition of class IV oocytes; (iii) fertilized eggs fatty acid composition.

Introduction

Zebrafish (*Danio rerio*) is a well-established experimental model for studies in the reproduction field since it is easily reared in laboratory conditions and, if compared to other teleost species, it reaches sexual maturity in a relatively short time [**1-4**]. In addition, over the last years, it gained a primary importance in assessing the role of specific dietary nutrients, such as fatty acids, in the reproductive processes of teleost [**5**,**6**]. Zebrafish is an asynchronous spawner, and, in females, the complete maturation of a single oocyte takes place in ~10 days [**1**,**2**,**4**]. Studies on reproductive mechanisms in zebrafish are also eased by a well-consolidated knowledge on oocyte development that is conventionally subdivided into five main stages [**4**,**7**]. Specifically, premeiotic oocyte progenitor cells, called oogonia, represent a continuous supply of new oocytes by multiple meiotic divisions. Once the oogonia enter meiotic division, they become primary oocytes. In this phase, oocytes initially reside in nests (Stage IA) and subsequently move from the original location and increase in size up to about 140 μm. Afterward, somatic cells are recruited with the formation of a definitive follicle (Stage IB) in which the oocyte is surrounded by an outer theca layer and an inner granulosa one. In Stage II, oocytes greatly increase in size up to a diameter of about 340 μm and membrane-limited

vesicles, called cortical alveoli, appear. In addition, in this stage, the oocyte lipid deposition initiate and induces the formation of oil droplets containing neutral lipids [**8**]. In Stage III (further divided in Stage IIIA and Stage IIIB), the uptake of a female-specific liver-derived glycolipoprotein, vitellogenin, supports the further follicle size increase up to a diameter of about 740 μm [**4**,**9**]. This process, called vitellogenesis, plays an important role in oocyte growth and fixes the transition from previtellogenic oocytes (Stages I and II) to vitellogenic ones (from Stage III onward). At the end of this phase (Stage IIIB), oocytes acquire maturational competence, by responding to maturation hormone stimuli. Later, in Stage IV, yolk vesicles containing vitellogenin begin to fuse with each other and oil droplets became more evident. Later, the nuclear envelope undergoes a breaking process, the ooplasm becomes opaque upon visual examination, and the follicle reaches the diameter of more than 740-760 μm [**9**]. Since maturation occurs in few hours, only a few follicles are usually found at this developmental stage in analysed zebrafish ovaries. The final maturation phase, traditionally described as Stage V, implies the complete fusion of the yolk vesicles and the separation of the mature oocyte from the follicular cells. Oocytes in this phase (diameter up to 1 mm) are rarely observed in zebrafish ovary, since they are ovulated into the ovarian lumen and rapidly spawned. Following ovulation, a typical postovulatory follicle (POF), composed of follicular cells (granulosa and theca cells) without the oocyte, remains in the ovary [**10**,**11**].

In natural and cultured fishes, a disruption in the reproductive process may often be observed [**12**]. In this case, females fail to complete maturation and ovulation due to the degeneration of vitellogenic oocytes before ovulation in a process known as follicular atresia [**13**,**14**]. A number of stressors, including food availability and quality, are known to potentially interfere with oogenesis and induce atresia of ovarian follicles [**15-19**], and should be studied in a deeper way when testing new aquafeed ingredients. The ongoing process of oocyte maturation is one of the most metabolic demanding activities in fish and the quality of maternal nutrition is of primary importance for the appropriate allocation of macromolecules into the oocytes and their maturation [**20-22**]. Nutritional imbalances are well known to be detrimental for oocyte development [**23**] and, for this reason, investigating whether a diet is able to correctly sustain fish reproduction or not is of primary importance.

Over the last decades, several dietary formulations have been adopted for zebrafish and some of them are now available in the market [**24-26**]. All these formulations mainly contain fish meal (FM) and fish oil (FO) because of their high nutritional value in terms of proteins, amino acid profile, and lipids. However, the European Community has recently addressed attention and investments in the search of alternative and more sustainable ingredients for aquafeed formulation. According to the EC Directive number 2008/98 on the optimization of waste management, the European Community encourages efforts in finding more responsible, sustainable, and innovative aquafeed ingredients

based on the circular economy concept, without affecting fish needs, welfare, and reproduction [**25-27**]. On this regard, recent studies explored the use of insect meal for aquafeed formulation, and several studies have already been performed on zebrafish [**28-30**]. Insects show several advantages, since most of them can be cultured on land produced by-products [**31-34**] and have a low environmental footprint in terms of land use, water consumption, and CO_2 production [**35-37**].

The interest toward insects as aquafeed ingredients is also due to their amino acidic profile that, for many insect species, matches the nutritional requirement of fish. Conversely, fatty acids profile of insects is considerably different from that of FO, being typically rich in saturated fatty acids (SFA) and poor in polyunsaturated ones (PUFA) [**38**,**39**]. Dietary fatty acid profile is a crucial aspect for maternal nutrition, playing an important role during oocyte maturation [**40**] and it has been demonstrated that especially an inappropriate PUFA intake can directly affect reproductive processes in teleost fish [**41-43**]. While several recent studies showed the possibility to partially replace FM and FO with insect meal during zebrafish culture without affecting fish growth and welfare [**44**], no information about the effects of these novel ingredients on reproduction is so far available.

In this study, female zebrafish were fed, for 12 months, a control diet based on FM and FO and two experimental diets, including two dietary inclusions of full-fat Black Soldier Fly (*Hermetia illucens*; BSF) prepupae meal (25 and 50 % respect to FM). The effects of these two experimental diets on reproduction in females were investigated through a multidisciplinary approach. The growth and the gonadosomatic index (GSI) of females were analysed and spawned and fertilized eggs as well as the hatching rate were evaluated to investigate reproductive performances.

Fatty acids composition of adult female carcasses and fertilized eggs was analysed through gas chromatography, while real-time quantitative PCR was used to evaluate the expression of genes involved in fish lipid metabolism in the liver. Finally, ovaries were subjected to: (i) histological analysis to assess oocyte maturation stages and the oil droplet percentage in class IV oocytes; (ii) spectroscopic analysis by Fourier Transform Infrared Microspectroscopy (FTIRM) to analyse the macromolecular composition of class IV oocytes.

Methods

Ethics

All procedures involving animals were conducted in line with Italian legislation on experimental animals and were approved by the Ethics Committee of the Università Politecnica delle Marche (Ancona, Italy; 84/94-A). Optimal rearing conditions (see further section for details) were applied throughout the study, and all efforts were made to minimize animal suffering by using an anaesthetic (MS222; Merck KGaA, Darmstadt, Germany).

Fish diet production

Diets were prepared according to Zarantoniello et al. [**29**] (chapter 2.1.). Specifically, two experimental diets, with increasing dietary substitution of FM with full-fat BSF prepupae meal (25 and 50 %, respectively) were tested in this study and compared to a FM-based diet, considered the control. BSF prepupae were purchased from a commercial company (Smart Bugs s.s. Company, Ponzano Veneto, Italy) and were reared on a substrate composed by corn meal, fruit, and vegetable mixture (50:50). Once collected, BSF prepupae were frozen (-80 °C), freeze-dried, and minced using liquid nitrogen.

Experimental diets (Table 1) were formulated to be isonitrogenous, isolipidic and isoenergetic by replacing 25 (BSF25) and 50 % (BSF50) of the FM/FO of the control diet with full-fat BSF prepupae meal, respectively.

Table 1. Ingredient composition, proximate analysis and gross energy content of the test diets

	Control	**BSF25**	**BSF50**
Ingredients (g/kg)			
Fish meal, Chile, super prime	420	315	210
Peas, protein concentrate	55	78	100
Hermetia illucens meal	0	105	210
Wheat gluten meal	55	78	100
Wheat flour	290	268	255
Fish oil	70	40	28
Palm oil	70	75	56
Min. & Vit. Supplement [1]	20	20	20
Binder	20	20	20
L-methionine	0	1	1
Proximate composition (%)			
Moisture	4.20±0.03	5.50±0.18	5.30±0.42
Crude protein	40.0±0.5	40.2±0.4	41.1±0.1
Crude lipids	18.6±0.1	17.7±0.2	17.0±0.1
Ash	14.2±0.2	14.1±0.3	12.2±0.6
N-free extractive	23.0±0.3	22.5±0.6	24.4±1.0
Gross Energy (MJ/kg)	22.1±0.1	22.3±0.1	21.3±0.1

[1]Composition of mineral mix (g/kg diet): Ca HPO_4 *$2H_2O$, 27.5; K_2HPO_4, 19.0; NaCl, 6.1; MgO, 2.0; $FeCO_3$, 1.75; KI, 0.15; ZnO, 0.11; MnO, 0.07; $CuSO_4$, 0.02; sodium selenite, 0.002. Composition of vitamin mix (mg/kg diet): thiamine HCl, 40; riboflavin, 40; pyridoxine HCl, 40; cyanocobalamin, 0.2; niacin, 300; calcium pantothenate, 100; folic acid, 5; biotin, 3; choline chloride, 5000; myo-inositol, 1000; ascorbic acid, 2000; a-tocopheryl acetate, 250; menadione, 90; vit. A retinyl palmitate, 40,000 IU/kg diet; vit. D3 cholecalciferol,2500 IU/kg diet.

All the diets were sieved to obtain a different granulometry as a function of fish size development (as reported in Feeding Schedule section). Diets (in triplicate subsamples) were analysed for proximate composition and gross energy content measured by an adiabatic bomb calorimeter (IKAC7000; Werke GmbH and Co., Staufen, Germany) [**44**].

Fish

Zebrafish (*Danio rerio*) used in this experiment were obtained from a pathogen-free AB broodstock (self-produced and that undergoes regular veterinary checks during the year) as described in Santangeli et al. [**45**]. Embryos were maintained for 48 h in a Tecniplast System (Varese, Italy), subjected to the following conditions: 28 °C temperature, pH 7.0, NH_3 and NO_2^- concentrations < 0.01 mg/L, NO_3^- concentration < 10 mg/L, and photoperiod 14L/10D. After this period, embryos were gently collected, counted, and randomly divided into three experimental groups (in triplicate) according to the three test diets.

Experimental design

Zebrafish larvae were initially reared in 9 tanks (20 L, 3 tanks per experimental group with 500 fish per tank, 1500 per dietary group). The water in larval tanks (same chemical-physical characteristics of broodstock tank) was gently replaced ten times a day by a dripping system [**46**]. The sides of the tanks were covered with black panels to reduce light reflection [**47**]. After 30 days post fertilization (dpf), fish of each tank were transferred to bigger tanks (100 L; nine in total; three per each dietary group), each equipped with mechanical and biological filtration (Panaque, Rome, Italy), and fed the same diets for 12 months [**48**]. After 12 months, fish were spawned (see details in further section) and part of the females was collected, anesthetized with a lethal dose of MS222 (1 g/L; Merck KGaA), and the liver, ovary, and carcass (whole fish deprived of internal organs) were sampled and properly stored for further analyses.

Feeding schedule

Starting from 5 dpf to 12 months, fish were fed as follows: zebrafish fed FM/FO diet (Control); zebrafish fed the diet including 25% of full-fat BSF prepupae meal (BSF25); and zebrafish fed the diet including 50% of full-fat BSF prepupae meal (BSF50). Feed particle sizes were < 100 µm from 5 to 15 dpf, 101-200 µm from 16 to 30 dpf, 201-400 µm from 31 to 60 dpf, and 401-600 µm from 61 dpf until the end of the experiment. Zebrafish were fed the experimental diets (2% body weight; BW) twice a day (total feed per day 4% BW) and, in addition, from 5 to 10 dpf, all groups were fed (one feeding in the morning) the rotifer *Brachionus plicatilis* (5 ind/mL) according to Lawrence et al. [**49**].

Growth and GSI

For growth measurements, 10 female zebrafish per tank (30 per dietary group) were randomly collected at the end of the experiment (12 months) and individually measured and weighed. The standard length was determined by a sliding calliper (precision: 0.1 mm) and wet weight by an

analytical balance (precision: 0.1 mg). Furthermore, ovaries from these fish (30 per dietary group) were individually weighted and the GSI was estimated, using the following formula: (ovary weight/ zebrafish BW) × 100.

Egg collection and hatching rate

For the egg collection, 12-month-old male and female zebrafish were randomly transferred to breeding tanks (Tecniplast System). Specifically, five females and seven males were daily transferred to the breeding tanks (3 per tank; 9 per experimental group) for spawning and egg collection, over a 10-day period. From each breading tank (about 20-30min after the onset of light), eggs were collected. Eggs were initially observed and counted under a stereomicroscope for the evaluation of total spawned eggs. Only fertilized eggs that presented a well-developed blastodisc at 3 h after fertilization were properly stored for further analyses and used for the hatching rate calculation [hatching rate = (hatched eggs/total fertilized eggs) × 100]. Fertilized eggs were transferred to 10 cm diameter Petri dishes containing E3 medium (5 mM NaCl, 0.17 mM KCl, 0.33 mM $CaCl_2$, and 0.33 mM $MgSO_4$, to pH 7.0) and incubated at 28 °C. More specifically, for the hatching rate evaluation, 150 fertilized eggs (in triplicate; 450 eggs per dietary group in total) from each breeding tank were randomly selected and distributed among Petri dishes (50 fertilized eggs per dish) containing E3 medium and then maintained in the incubator at 28 °C for 5 days post fertilization. Hatched larvae were finally counted.

Lipid extraction and fatty acids analysis

Twelve-month-old female carcasses (3 female zebrafish per tank; 9 per dietary group) and just fertilized eggs (3 pools of 300 eggs per tank; 9 pools per dietary group) were analysed for lipid content and fatty acids composition. Samples were minced, homogenized (homogenizer MZ 4110; DCG Eltronic, Monza, Italy), freeze-dried (Edwards EF4, Crawley, Sussex, England), and lipid extraction was carried out on lyophilized powders following a microwave-assisted extraction [**50**,**51**]. Fatty acids methyl esters (FAME) were prepared according to Truzzi et al. [**52**] using the methyl ester of nonadecanoic acid (19:0; Dr. Ehrenstorfer GmbH, Augsburg, Germany) as internal standard. FAME were determined by an Agilent-6890 GC System (Milano, Italy) coupled to an Agilent-5973N quadrupole Mass Selective Detector. A CPS ANALITICA CC-wax-MS (30 m × 0.25 mm inner diameter and 0.25 μm film thickness) capillary column was used to separate FAME. Instrumental conditions for the studied matrices were set up, according to Truzzi et al. [**53**,**54**]. For each analysed aliquot of sample, at least three runs were performed on the GC-MS.

Histological analyses

Ovaries collected from 5 different female zebrafish per tank (15 per dietary group) were used for the histological analysis and analysed according to Migliaccio et al. [**55**]. Briefly, after fixation in Bouin's solution (Merck KGaA; 4 °C for 24 h), samples were washed three times, for 10 min each, with ethanol (70%) and stored in the same ethanol solution. Samples were then dehydrated in increasing ethanol solutions (80, 95 and 100%), washed with xylene (Bio-Optica, Milano, Italy), and embedded in paraffin (Bio-Optica). Solidified paraffin blocks were cut with a microtome (Leica RM2125 RTS; Leica) and each ovary was fully sectioned. Sections (5 μm) were stained with Mayer hematoxylin and eosin Y (Merck KGaA) and observed using a Zeiss Axio Imager.A2 (Zeiss, Oberkochen, Germany) microscope to count the number of previtellogenic, class III, class IV, and atretic oocytes and POF based on their morphological features [**55**]. Images were acquired by means of a combined colour digital camera Axiocam 503 (Zeiss) and all the sections were analysed. Oocyte count was performed by using ZEN 2.3 lite Software (Zeiss). To minimize repetition during the count, oocytes were marked in each section. Results were reported as the percentage of previtellogenic, class III, class IV and atretic oocytes and POF on the total of oocytes counted. To further evaluate the histological features of class IV oocytes, the percentage of ooplasm area occupied by oil droplets (oil droplets area = ODA) at this stage was analysed. For the ODA analysis, class IV oocytes of each dietary group (n = 9 per group) were analysed. Specifically, histological pictures of class IV oocytes with a diameter ranging from 700 to 740 μm were acquired by mean of ZEN 2.3 lite Software (Zeiss), while the area outside the oocyte was not acquired to avoid measurements on false positive areas (see Fig. 8b). Images were then analysed by means of the ImageJ software. A homogeneous threshold was used to evaluate the white area in ooplasm (ODA), and data were reported as the mean ± SD of the observations.

Fourier Transform Infrared Microspectroscopy

FTIRM measurements were carried out by means of a Bruker Invenio interferometer coupled with a Hyperion 3000 Vis-IR microscope and equipped with a MCT (Hg-Cd-Te) detector operating at liquid nitrogen temperature (Bruker Optics, Ettlingen, Germany). Ovaries were collected from zebrafish specimens (2 per tank; 6 per dietary group) and stored at -80 °C until they were cut by means of a cryomicrotome. For each sample, three sections (10 μm thick) were cut at 2mm distance from each other. Sections were immediately deposited without any fixation process onto CaF_2 optical windows (1 mm thick and 13 mm in diameter), and then air-dried for 30 min [**56**]. Sections were observed by means of a 15·condenser/objective, to select class IV oocytes to be analysed by FTIRM. On the inner compartment (excluding oolemma, nucleus, and zona radiata) of each selected oocyte, ~20 infrared

(IR) spectra were acquired in transmission mode in the mid-infrared (MIR) range (4000-800 cm^{-1}; spectral resolution 4 cm^{-1}; spatial resolution 50 × 50 µm; 128 scans) [**57**,**58**]. Before each acquisition, the background spectrum was acquired on a clean portion of the CaF_2 optical window. Raw IR spectra were pre-processed by Atmospheric Compensation (to correct for the atmospheric contributions of carbon dioxide and water vapor) and Vector Normalization (applied on the full frequency range, to avoid any artifact due to section thickness variations) routines (OPUS 7.5 software package; Bruker Optics). IR spectra were then converted in second derivative mode (Savitzky-Golay filter, 9-point smoothing) and submitted to Principal Component Analysis (PCA) both alone and coupled with Linear Discriminant Analysis (PCA-LDA; OriginPro 2018b software; OriginLab Corporation, Northampton, MA). On pre-processed IR spectra, meaningful band area ratios were calculated, as ratio between the integrated areas of specific IR bands. Instead, the integrated areas of the following spectral regions were calculated (Integration routine, Mode B, OPUS 7.5 software package): 3050-3000 cm-^{1} (representative of =CH groups in unsaturated lipid alkyl chains, named CH); 3000-2824 cm^{-1} (representative of CH2 and CH3 groups in lipid alkyl chains, named LIP); 1767-1710 cm^{-1} (representative of C=O groups in fatty acids, named FA); 1710-1478 cm^{-1} (representative of Amide I and II bands of proteins, named PRT); 1478-1426 cm^{-1} (representative of CH2 groups in saturated lipid alkyl chains, named CH2); 1426-1358 cm^{-1} (representative of COO^- groups in amino acids, named COO); 1290-1188 cm^{-1} (representative of phosphate groups, named PH); 1188-1141 cm^{-1} (representative of COH groups in glycosylated compounds, named COH), and 1141-992 cm^{-1} (representative of phosphates and carbohydrates, named PH-CARBO).

RNA extraction and cDNA synthesis

Liver samples from 5 different female zebrafish from each tank (15 fish per dietary group) were sampled and then stored at -80 °C for molecular analysis. Analyses were performed according to Olivotto et al. [**59**]. Briefly, total RNA extraction from each sample was optimized using RNAzol® RT reagent (R4533; Merck KGaA) following the manufacturer's instructions. Total RNA was eluted in 20 µL of RNase-free water (Qiagen, Venlo, Holland). Final RNA concentration was determined by the NanoPhotometer® P-Class (Implen, München, Germany). RNA integrity was verified by GelRed™ staining of 28S and 18S ribosomal RNA bands on 1% agarose gel. RNA was stored at -80 °C until use. Finally, 2 µg of RNA were used for complementary DNA (cDNA) synthesis, employing the High Capacity cDNA Reverse Transcription Kit (Bio-Rad, Hercules, CA, USA) following the manufacturer's instructions.

Real-time PCR

PCRs were performed with SYBER Green in an iQ5 iCycler thermal cycler (both from Bio-Rad) following Zarantoniello et al. [**39**]. The thermal profile for all reactions was: 3 min at 95 °C, followed by 45 cycles of 20 s at 95 °C, 20 s at the specific annealing temperature of each primer (Table 2) and 20 s at 72 °C. Fluorescence was monitored at the end of each cycle. Melting curve analysis showed a single pick in all cases. Relative quantification of the expression of genes involved in long-chain PUFA biosynthesis (fatty acid elongase 2, *elovl2*; fatty acid elongase 5, *elovl5;* fatty acid desaturase 2, *fads2*) was performed. Actin-related protein 2/3 complex, subunit 1A (*arpc1a*) and ribosomal protein, large, 13 (*rpl13*) were used as internal standards in each sample to standardize the results by eliminating variation in mRNA and cDNA quantity and quality. Amplification products were sequenced, and homology was verified. No amplification product was detected in negative controls and no primer-dimer formation was found in control templates. Data were analysed using the iQ5 optical system software version 2.0, including Genex Macro iQ5 Conversion and Genex Macro iQ5 files (all from Bio-Rad). Modification of gene expression was reported with respect to controls. Primer sequences were designed using Primer3 (210 v. 0.4.0) starting from zebrafish sequences available in ZFIN (for details, please see Table 2).

Table 2. Sequences, Zebrafish Information Network (ZFIN) IDs, and annealing temperature (A.T.) of the primers used for the present study.

Gene	Forward Primer (5'-3')	Reverse Primer (5'-3')	ZFIN IDs	A. T. (°C)
elovl2	CACTGGACGAAGTTGGTGAA	GTTGAGGACACACCACCAGA	ZDB-GENE-011212-1	60
elovl5	TGGATGGGACCGAAATACAT	GTCTCCTCCACTGTGGGTGT	ZDB-GENE-040407-2	60
fads2	CATCACGCTAAACCCAACA	GGGAGGACCAATGAAGAAGA	ZDB-GENE-011212-1	60
arpc1a	CTGAACATCTCGCCCTTCTC	TAGCCGATCTGCAGACACAC	ZDB-GENE-040116-1	60
rpl13	TCTGGAGGACTGTAAGAGGTATGC	AGACGCACAATCTTGAGAGCAG	ZDB-GENE-031007-1	59

Statistical analyses

Data obtained were analysed by one-way analysis of variance (ANOVA), with diet as the explanatory variable. All ANOVA tests were followed by Tukey's post hoc test. The statistical software package Prism 6 (GraphPad Software) was used. Significance was set at $p < 0.05$ and all the results are presented as mean ± SD. In addition, IR spectra were then converted in second derivative mode (Savitzky-Golay filter, 9-point smoothing) and submitted to PCA both alone and coupled with LDA (OriginPro 2018b software). PCA was first used to reduce redundant information from the spectral dataset, describing each spectrum with a subset of selected principal components (PCs), explaining 95% of cumulative variance; then, the reduced spectra were used as input variables for LDA [**60**].

Results

Growth and GSI

As reported in Figure 1a, the standard length of zebrafish adult females from BSF25 and BSF50 groups (27.7 ± 4.0 and 26.7 ± 2.3 mm for BSF25 and BSF50, respectively) was significantly lower compared to Control group (32.2 ± 3.0 mm). Conversely, as concern both wet weight (0.7 ± 0.1, 0.7 ± 0.1, and 0.6 ± 0.1 g for Control, BSF25, and BSF50, respectively; Fig. 1b) and GSI (10.8 ± 0.8, 10.7 ± 1.5, and 10.0 ± 2.3 % for Control, BSF25, and BSF50, respectively; Fig. 1c) no significant differences were detected among the experimental groups.

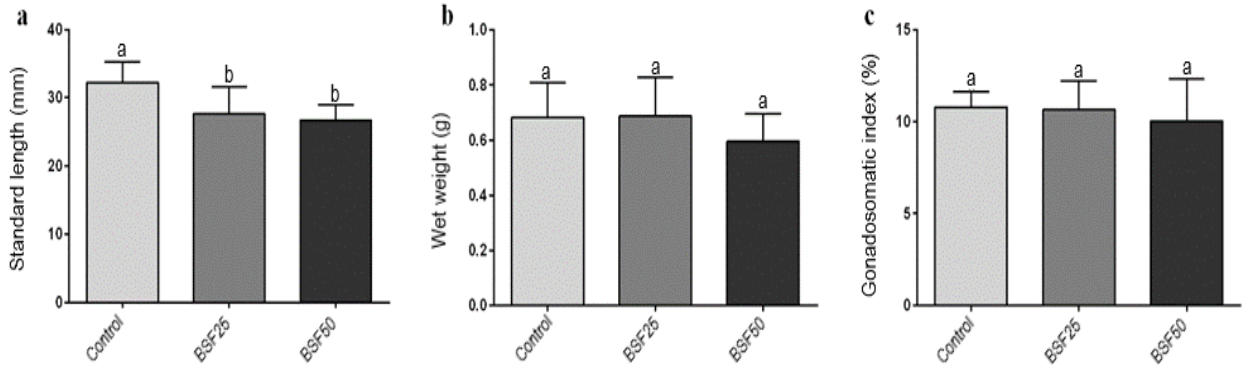

Figure 1. Biometric measurements of 12-month-old female zebrafish. (**a**) standard length, (**b**) wet weight, and (**c**) gonadosomatic index. Different letters indicate statistically significant differences among experimental groups ($p < 0.05$). Values are presented as mean ± SD (n = 15). Zebrafish fed diet based on FM (Control) and diets with 25 (BSF25) or 50 % (BSF50) replacement of FM with full-fat BSF prepupae meal.

Spawned eggs and hatching rate

As reported in Table 3, a significant reduction of both daily and total spawned eggs was observed in BSF50 group (81 ± 42 and 811 ± 36 for daily and total spawned eggs, respectively) compared to the other experimental groups (192 ± 37 and 190 ± 48 daily spawned eggs for Control and BSF25, respectively; 1892 ± 53 and 1903 ± 94 total spawned eggs for Control and BSF25, respectively), that did not show significant differences between them. As regard to the hatching rate (Fig. 2), no significant differences were detected among the experimental groups (74.4 ± 2.6, 75.3 ± 2.6 and 73.2 ± 2.2 % for Control, BSF25 and BSF50, respectively).

Table 3 Number of total and daily spawned eggs per breeding tank in a 10-day period.

N. of spawned eggs	Control	BSF25	BSF50
Daily	192 ± 37 [a]	190 ± 48 [a]	81 ± 42 [b]
Total	1892 ± 53 [a]	1903 ± 94 [a]	811 ± 36 [b]

Different letters indicate statistically significant differences among experimental groups ($p < 0.05$). Values are presented as mean ± SD (n = 9). Zebrafish fed diet based on FM (Control) and diets with 25 (BSF25) or 50 % (BSF50) replacement of FM with full-fat BSF prepupae meal.

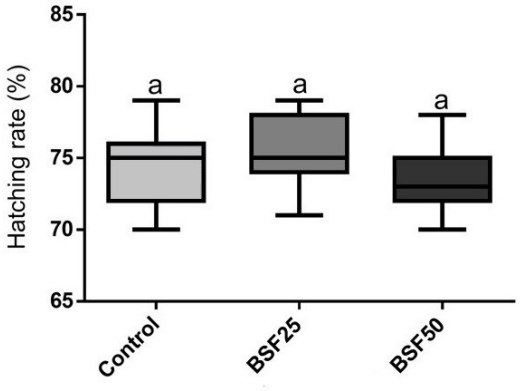

Figure 2. Hatching rate (%). Different letters indicate statistically significant differences among experimental groups ($p < 0.05$). Values are presented as mean ± SD (n = 9). Zebrafish fed diet based on FM (Control) and diets with 25 (BSF25) or 50 % (BSF50) replacement of FM with full-fat BSF prepupae meal.

Fatty acids content and composition

Adult females' carcasses. The fatty acids content (as % of total fatty acids) of 12-month-old female zebrafish fed the different diets was affected by increasing dietary inclusion levels of full-fat BSF prepupae meal (Fig. 3a). Specifically, a significant increase of SFA content was detected in BSF25 and BSF50 groups (27.5 ± 0.4 and 33.7 ± 0.4 %) with respect to Control (25.8 ± 0.4 ±). Regarding monounsaturated fatty acids (MUFA) and n6, both BSF25 (40.3 ± 0.7 and 15.9 ± 0.1 % for MUFA and n6, respectively) and BSF50 (39.0 ± 0.4 and 16.3 ± 0.2 %, for MUFA and n6, respectively) groups showed a significantly higher percentage with respect to Control (35.2 ± 0.4 and 14.6 ± 0.3 % for MUFA and n6, respectively), but did not show significant differences between them. The increasing inclusion levels of full-fat BSF prepupae meal in the diets resulted in a significant decrease of both PUFA (39.1 ± 0.6, 32.3 ± 0.4 and 27.3 ± 0.3 % for Control, BSF25 and BSF50) and n3 content (24.4 ± 0.5, 16.4 ± 0.4 and 11.1 ± 0.2 % for Control, BSF25 and BSF50). Differently, considering n9 content, BSF25 and BSF50 groups (32.2 ± 0.7 and 30.8 ± 0.4 %, respectively) evidenced significantly higher percentages respect to Control (25.9 ± 0.3%). Finally, as presented in Figure 3b, the higher was the dietary BSF full-fat prepupae inclusion, the higher was the n6/n3 ratio (0.6 ± 0.1, 1.0 ± 0.1, 1.5 ± 0.1 for Control, BSF25 and BSF50, respectively). Considering SFA composition in the three experimental groups (Table 4), the predominant one was the palmitic acid (16:0), followed by stearic acid (18:0). Both these fatty acids did not present significant differences among the experimental groups. BSF50 group showed a significantly higher content of lauric acid (12:0) with respect to BSF25 and Control groups. The most represented MUFA was oleic acid (18:1n9) and its content was significantly higher in BSF25 and BSF50 groups with respect to Control. Finally, linoleic acid (18:2n6) was the predominant PUFA, with significantly higher percentages in BSF25 and BSF50 fish with respect to Control. Groups fed BSF-based diets showed a significantly lower eicosapentaenoic acid (EPA; 3.0 ± 0.3 and 2.3 ± 0.4 % for BSF25 and BSF50, respectively) and docosahexaenoic acid

(DHA; 8.3 ± 0.9 and 4.6 ± 1.1 % for BSF25 and BSF50, respectively) content than Control (6.0 ± 0.8 and 14.3 ± 0.9 % for EPA and DHA, respectively).

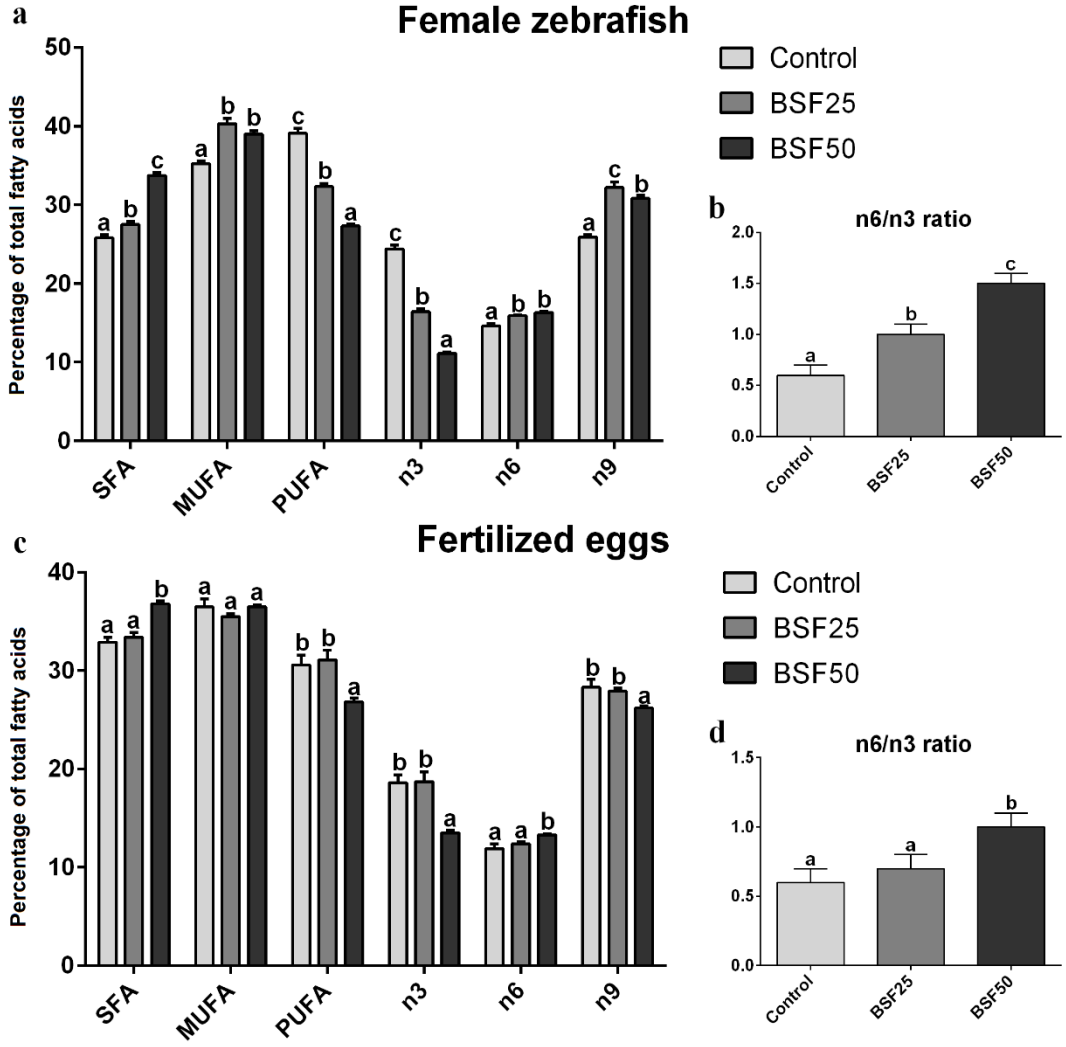

Figure 3. Content of SFA, MUFA, and PUFA (as % of total fatty acids) and contribution of omega 3 (n3), omega 6 (n6), and omega 9 (n9) fatty acids to lipid profile. (**a**,**b**) Twelve-month-old zebrafish females' carcasses (Control, BSF25, and BSF50); (**c**,**d**) just fertilized eggs obtained from Control, BSF25, and BSF50 fish groups. Different letters indicate statistically significant differences among experimental groups ($p < 0.05$). Values are presented as mean ± SD (n = 9). Zebrafish fed diet based on FM (Control) and diets with 25 (BSF25) or 50 % (BSF50) replacement of FM with full-fat BSF prepupae meal. For fatty acids content of the diets, please see Zarantoniello et al. [**29**] (chapter 2.1).

Fertilized eggs. The fatty acids content (as % of total fatty acids) of just fertilized eggs from the three experimental groups is presented in Figure 3c. Considering SFA and n6, BSF50 group showed a significantly higher percentage (36.8 ± 0.3 and 13.3 ± 0.1 % for SFA and n6, respectively) with respect to Control (32.9 ± 0.5 and 11.9 ± 0.5 % for SFA and n6, respectively) and BSF25 (33.4 ± 0.5 and 12.4 ± 0.2 % for SFA and n6, respectively) groups. As concerns MUFA, no significant differences were detected among the experimental groups (36.5 ± 0.8, 35.5 ± 0.3, and 36.5 ± 0.2 %

Table 4. Fatty acids composition (% fatty acids methyl esters) of adult females' carcasses and fertilized eggs

	Female zebrafish			Fertilized eggs		
	Control	**BSF25**	**BSF50**	**Control**	**BSF25**	**BSF50**
10:0	n.d.	0.03±0.01 [a]	0.08±0.01 [b]	n.d.	0.04±0.01 [a]	0.1±0.01 [b]
12:0	0.2±0.1 [a]	4.4±0.4 [b]	8.9±1.1 [c]	0.3±0.1 [a]	2.0±0.2 [c]	1.6±0.1 [b]
13:0	0.03±0.01 [a]	0.02±0.01 [a]	0.03±0.01 [a]	0.01±0.01 [a]	0.01±0.01 [a]	0.02±0.01 [a]
14:0	2.7±0.1 [a]	2.8±0.2 [a]	4.2±0.6 [b]	1.7±0.1 [a]	2.3±0.1 [b]	3.1±0.2 [c]
14:1n5	0.1±0.1 [a]	0.3±0.1 [ab]	0.5±0.1 [b]	0.1±0.1 [a]	0.1±0.1 [a]	0.2±0.1 [a]
15:0	0.5±0.1 [a]	0.4±0.1 [a]	0.4±0.1 [a]	0.5±0.1 [a]	0.4±0.1 [a]	0.5±0.1 [a]
15:1n5	0.2±0.1 [a]	0.3±0.1 [a]	0.3±0.1 [a]	0.4±0.1 [a]	0.3±0.1 [a]	0.4±0.1 [a]
16:0	16.9±0.1 [a]	16.2±1.6 [a]	17.1±1.0 [a]	23.6±0.9 [ab]	22.0±0.1 [a]	24.3±1.0 [b]
16:1n9	1.0±0.1 [a]	1.3±0.3 [a]	1.3±0.4 [a]	2.4±0.1 [ab]	2.6±0.1 [b]	2.2±0.1 [a]
16:1n7	4.7±0.6 [a]	4.0±0.3 [a]	4.2±0.1 [a]	3.7±0.1 [a]	3.8±0.1 [a]	4.9±0.1 [b]
17:0	0.7±0.1 [b]	0.5±0.1 [ab]	0.4±0.1 [a]	0.6±0.1 [a]	0.5±0.1 [a]	0.7±0.1 [a]
17:1n7	0.7±0.1 [a]	0.6±0.1 [a]	0.5±0.1 [a]	0.6±0.1 [a]	0.6±0.1 [a]	0.8±0.1 [a]
18:0	4.4±0.3 [c]	2.9±0.1 [b]	2.4±0.1 [a]	6.1±0.2 [a]	6.1±0.1 [a]	6.3±0.2 [a]
18:1n9	23.4±1.9 [a]	29.9±2.2 [b]	28.8±1.4 [b]	24.2±0.2 [b]	24.4±0.2 [b]	23.1±0.4 [a]
18:1n7	3.5±0.2 [b]	2.9±0.2 [a]	2.6±0.1 [a]	3.5±0.1 [b]	2.8±0.1 [a]	4.0±0.1 [c]
18:2n6	11.7±0.1 [a]	13.2±0.8 [b]	13.2±0.2 [b]	8.5±0.1 [a]	8.8±0.1 [b]	8.9±0.1 [b]
18:3n6	0.3±0.1 [a]	0.1±0.1 [a]	0.1±0.1 [a]	0.2±0.1 [a]	0.4±0.2 [a]	0.2±0.1 [a]
18:3n3	3.6±0.3 [a]	4.7±1.3 [a]	3.9±0.8 [a]	3.0±0.1 [b]	3.0±0.2 [b]	1.2±0.1 [a]
20:0	0.2±0.1 [a]	0.1±0.1 [a]	0.1±0.1 [a]	0.2±0.1 [a]	0.1±0.1 [a]	0.1±0.1 [a]
20:1n9	0.3±0.1 [a]	0.4±0.2 [a]	0.3±0.1 [a]	1.6±0.1 [b]	0.8±0.1 [a]	0.9±0.1 [a]
20:2n6	0.6±0.1 [a]	0.6±0.1 [a]	0.7±0.1 [a]	1.1±0.1 [b]	0.7±0.1 [a]	0.9±0.1 [ab]
20:3n6	0.6±0.0 [a]	0.7±0.2 [ab]	1.0±0.1 [b]	1.5±0.1 [a]	1.5±0.1 [a]	1.8±0.1 [b]
20:4n6	1.4±0.2 [a]	1.3±0.3 [a]	1.4±0.2 [a]	0.8±0.1 [a]	1.0±0.1 [a]	1.4±0.2 [b]
20:3n3	0.5±0.2 [a]	0.4±0.1 [a]	0.3±0.1 [a]	1.0±0.1 [c]	0.6±0.1 [b]	0.3±0.1 [a]
20:5n3	6.0±0.8 [b]	3.0±0.3 [a]	2.3±0.4 [a]	4.2±0.2 [b]	4.5±0.1 [b]	2.8±0.1 [a]
22:0	0.15±0.02 [b]	0.09±0.02 [a]	0.07±0.02 [a]	0.05±0.01 [b]	0.03±0.01 [ab]	0.02±0.01 [a]
22:1n9	1.0±0.2 [b]	0.5±0.1 [a]	0.4±0.1 [a]	0.16±0.01 [c]	0.08±0.03 [b]	0.03±0.01 [a]
22:6n3	14.3±0.9 [b]	8.3±0.9 [b]	4.6±1.1 [a]	10.4±0.4 [b]	10.6±0.5 [b]	9.2±0.1 [a]
24:1n9	0.2±0.1 [a]	0.1±0.1 [a]	0.0±0.1 [a]	n.d.	n.d.	n.d.

For each matrix, mean within rows bearing different letters are significantly different ($p < 0.05$; $n = 9$). **Females' carcasses**: zebrafish fed diet based on FM (Control) and diets with 25 (BSF25) or 50 % (BSF50) replacement of FM with full-fat BSF prepupae meal. **Fertilized eggs**: obtained from Control, BSF25, and BSF50 fish groups. For fatty acids composition of the diets, please see Zarantoniello et al. [**29**] (chapter 2.1).

for Control, BSF25 and BSF50, respectively). Regarding PUFA, n3, and n9, BSF50 group (26.8 ± 0.4, 13.5 ± 0.3, and 26.2 ± 0.2 % for PUFA, n3 and n9, respectively) showed significantly lower percentages with respect to Control (30.6 ± 1.0, 18.6 ± 0.8, and 28.3 ± 0.8 % for PUFA, n3 and n9, respectively) and BSF25 group (31.1 ± 1.0, 18.7 ± 1.0, and 27.9 ± 0.3 % for PUFA, n3 and n9, respectively). Finally, as concerns the n6/n3 ratio (Fig. 3d), Control (0.6 ± 0.1) and BSF25 (0.7 ± 0.1) groups were characterized by a significantly lower value with respect to BSF50 group (1.0 ± 0.1). With regard to specific fatty acids composition (Table 4), the most relevant SFA in all the

experimental diets was the palmitic acid (16:0). Lauric acid (12:0) content was significantly higher in fertilized eggs spawned by fish fed BSF-based diets with respect to Control. Oleic acid (18:1n9) was the most abundant MUFA in eggs from all the dietary treatments. Finally, just fertilized eggs from BSF50 group showed a significantly lower EPA and DHA content (2.8 ± 0.1 and 9.2 ± 0.1 %, respectively) with respect to Control (4.2 ± 0.2 and 10.4 ± 0.4 % for EPA and DHA, respectively) and BSF25 (4.5 ± 0.1 and 10.6 ± 0.5 % for EPA and DHA, respectively) groups, which did not present significant differences between them.

Histological analysis of ovaries

Figure 4 shows representative histological sections of whole zebrafish ovaries. Histological analyses performed on ovary sections of Control, BSF25 and BSF50 groups showed all the different classes of oocytes (Fig. 5). In particular, for the oocyte count, oocytes were assigned at three developmental stages: (i) previtellogenic: oocytes with or without presence of cortical alveus (100-280 µm diameter; Fig. 5a,b); (ii) class III: oocytes with enlarged yolk vesicles and vitellin membrane (280-740 µm diameter; Fig. 5c); (iii) class IV: oocytes without nuclear envelope and opaque ooplasm (>740 µm diameter; Fig. 5d). Since class V oocytes are ovulated in few hours, they were rarely found in the analysed zebrafish ovaries. After ovulation, follicular cells (theca and granulosa cells) remain in the ovary and constitute a characteristic histological structure called POF, identified as collapsed empty follicles made of intact granulosa and theca cell layers, without oocyte (Fig. 5e). For this reason, we decided to exclude class V oocytes from the analysis and addressed our analysis on the percentage of POF number with respect to the total number of oocytes per ovary analysed. Atretic oocytes were observed in particular in the BSF50 group. Atretic oocytes were identified as described in Üçüncü, and Çakici [**61**] and were characterized by basal membrane disintegration, invagination and breakdowns of zona radiate, degeneration and absorption of ooplasm, and granulosa and theca cell hyperplasia (Fig. 6).

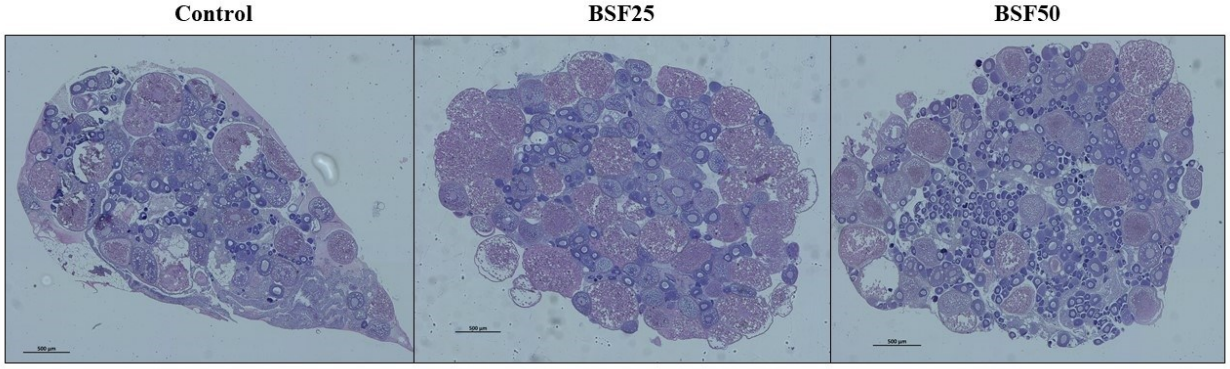

Figure 4. Example of histomorphology of zebrafish ovaries. Scale bars: 500 µm. Zebrafish fed diet based on FM (Control) and diets with 25 (BSF25) or 50 % (BSF50) replacement of FM with full-fat BSF prepupae meal.

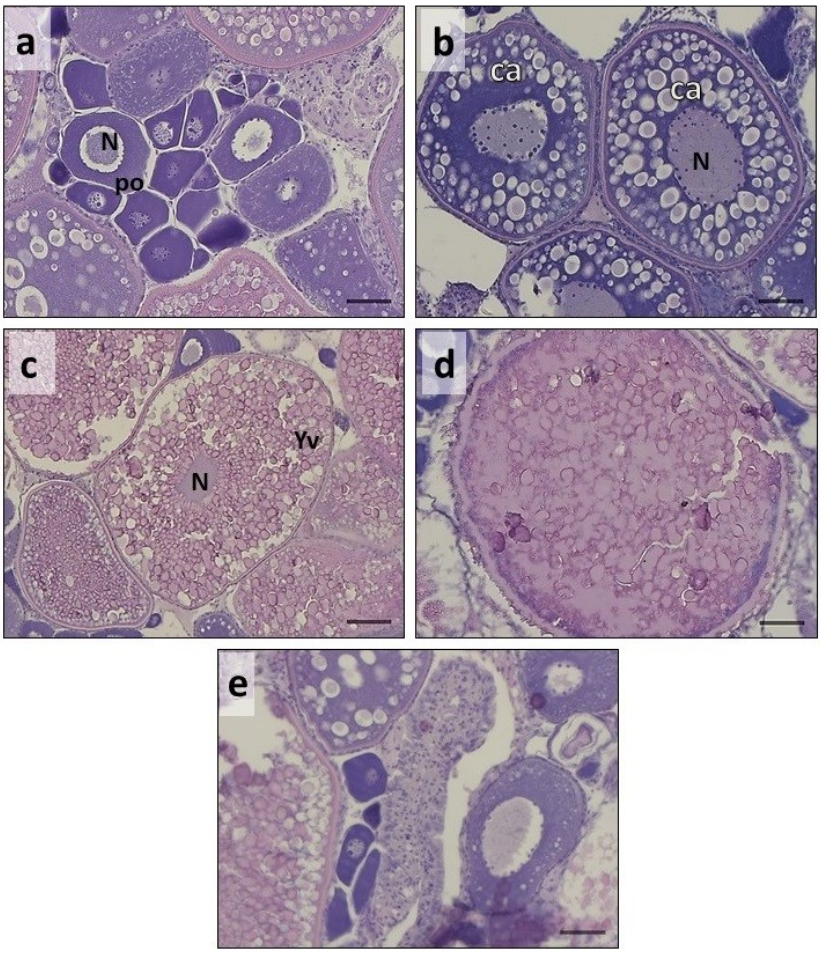

Figure 5. Oocyte developmental stages in zebrafish ovary. Previtellogenic stages: (**a**) oogonia and primary oocytes lying in nest; (**b**) previtellogenic oocytes with cortical alveoli. (**c**) oocyte at stage III with yolk vesicles; (**d**) oocyte at stage IV; (**e**) POF. Letters: po, primary oocytes; N, nucleus ca, cortical alveoli; Yv, yolk vesicles. Scale: (**a**,**b**) 50 µm, (**c**,**d**) 100 µm, (**e**) 200 µm.

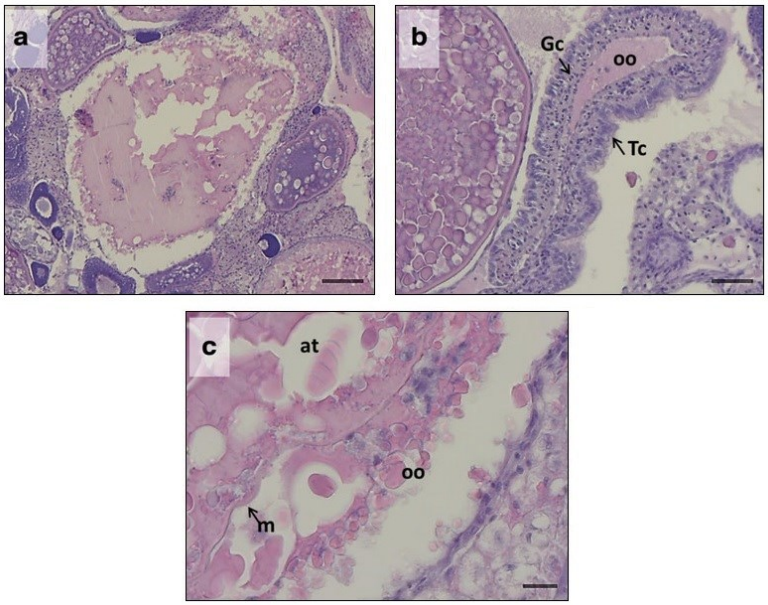

Figure 6. Atretic oocyte. (**a**) Low magnification of an atretic oocyte; (**b**) iperplastic follicular theca (Tc) and granulosa (Gc) cells surrounding degenerating ooplasm (oo) of an atretic oocyte; (**c**) high magnification showing corrugated oocyte membrane (m), atretic zones (at), and degenerated expelled ooplasm. Scale: (**a**) 200 µm, (**b**) 100 µm, (**c**) 20 µm.

Figure 7 shows the frequency of the different oocyte classes. BSF50 group showed a significantly higher percentage of previtellogenic oocytes (86.5 ± 4.5 %) with respect to both Control (78.5 ± 3.5 %) and BSF25 (76.5 ± 3.5 %) groups, which did not evidence significant differences between them. Conversely, as concerns class III oocytes, Control and BSF25 groups showed a significantly higher percentage (19.5 ± 3.5 and 20.5 ± 1.5 %, respectively) than BSF50 group (4.5 ± 2.5 %). Considering class IV oocytes (1.5 ± 0.5, 1.5 ± 0.5 and 1.5 ± 0.5 % for Control, BSF25 and BSF50, respectively) and POF (1.8 ± 0.8, 1.9 ± 0.9 and 1.8 ± 0.8 % for Control, BSF25 and BSF50, respectively), no significant differences were detected among the experimental groups. On the contrary, a significant increase of atretic oocytes was observed in BSF50 group (8.5 ± 1.5 %) compared to the Control (0.5 ± 0.5 %) and BSF25 (1.0 ± 0.5 %) groups.

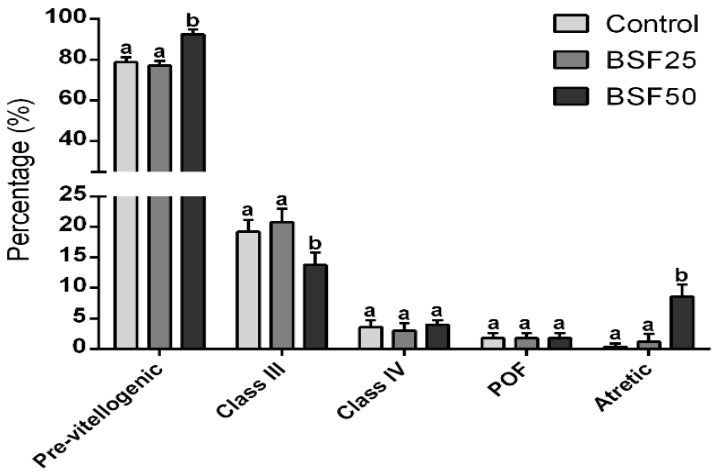

Figure 7. Percentage of previtellogenic, class III, class IV, and class V oocytes, POF and atretic oocytes. Different letters indicate statistically significant differences among experimental groups ($p < 0.05$). Values are presented as mean ± SD ($n = 15$). Zebrafish fed diet based on FM (Control) and diets with 25 (BSF25) or 50 % (BSF50) replacement of FM with full-fat BSF prepupae meal.

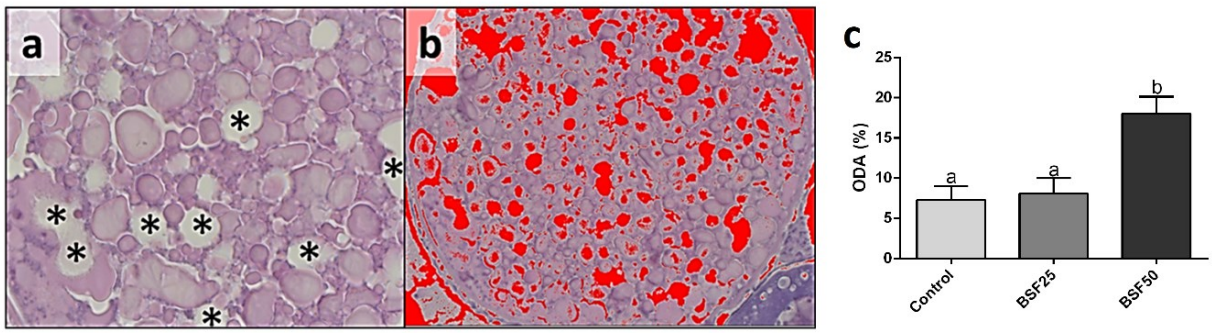

Figure 8. Percentage of the area occupied by lipid droplets in class IV oocytes. (**a**) Histological representative section of a class IV oocyte with oil droplets (*); (**b**) example of analysis of the area occupied by oil droplets (ODA) performed with ImageJ software; (**c**) ODA percentage. Results are representative of class IV oocyte sections (n = 3/ovary) from three different ovary (total 9/group) \and expressed in percentage of ODA on the total ooplasm. Different letters indicate statistically significant differences among experimental groups ($p < 0.05$). Values are presented as mean ± SD. Zebrafish fed diet based on FM (Control) and diets with 25 (BSF25) or 50 % (BSF50) replacement of FM with full-fat BSF prepupae meal.

Finally, representative images of the analysis of the area occupied by lipid droplets (ODA) on histological sections of class IV oocytes are shown in Figure 8a,b. Results evidenced a significantly higher ODA in BSF50 group oocytes (20 ± 2.7 %) compared to Control (6.3 ± 1.7 %) and BSF25 (8.1 ± 9.1 %) groups (Fig. 8c).

FTIRM analyses

The inner compartment (excluding oolemma, nucleus, and zona radiata) of class IV oocytes from 12-month-old zebrafish females feeding the experimental diets was investigated by FTIRM spectroscopy. In Figure 9a, the average IR spectrum of a class IV oocyte is shown. Specific bands of the biochemical components of the sample were detected: 3015 cm^{-1} (stretching mode of =CH groups in lipid alkyl chains); 2959, 2927, and 2856 cm^{-1} (symmetric and asymmetric stretching modes of CH2 and CH3 groups in lipid alkyl chains); 1739 cm^{-1} (stretching mode of C=O groups in fatty acids); 1652 and 1541 cm^{-1} (Amide I and II bands of proteins); 1455 cm^{-1} (bending mode of CH2 groups, mainly in lipids); 1396 cm^{-1} (stretching mode of COO- groups in amino acids); 1237 cm^{-1} (asymmetric stretching mode of phosphate groups); 1156 cm^{-1} (stretching mode of COH groups in glycosylated compounds); 1084 cm^{-1} (symmetric stretching mode of phosphate groups and stretching modes of C-O and C-C bonds in carbohydrates). IR spectra of Control, BSF25, and BSF50 groups were submitted to PCA and PCA-LDA analyses to highlight the spectral features of this cell compartment in relationship with the different diets (Fig. 9b-e). With regard to PCA scores plot, no segregation pattern was found among all the experimental groups (Fig. 9b), even if the PC1 and PC2 loading spectra pinpointed changes in the regions related to lipids (zone 1), fatty acids (zone 2), and proteins (zone 3) (Fig. 9c). Conversely, PCA-LDA displayed a complete separation of all the three experimental groups (Fig. 9d), with LD1 and LD2 representing the first and second linear discriminant functions obtained by the canonical variable scores of PCA-LDA. In particular, LD1 showed to discriminate between Control and the other two groups, while LD2 allowed to distinguish between BSF25 and BSF50 spectra. The one-dimensional score plot of LD1 and LD2 displayed in Figure 9e confirmed the importance of the two linear discriminant variables in distinguishing the three experimental groups.

To investigate the biochemical composition of the inner compartment of Control, BSF25, and BSF50 class IV oocytes, the following band area ratios were calculated and statistically analysed (Fig. 10): LIP/CELL (relative amount of overall lipids); CH2/CELL (relative amount of SFA); CH/CELL (relative amount of unsaturated fatty acids); FA/CELL(relative amount of overall fatty acids); PRT/CELL (relative amount of overall proteins); COO/CELL (relative amount of COO- in amino acids); PH/CELL (relative amount of phosphates); COH/CELL (relative amount of glycosylated

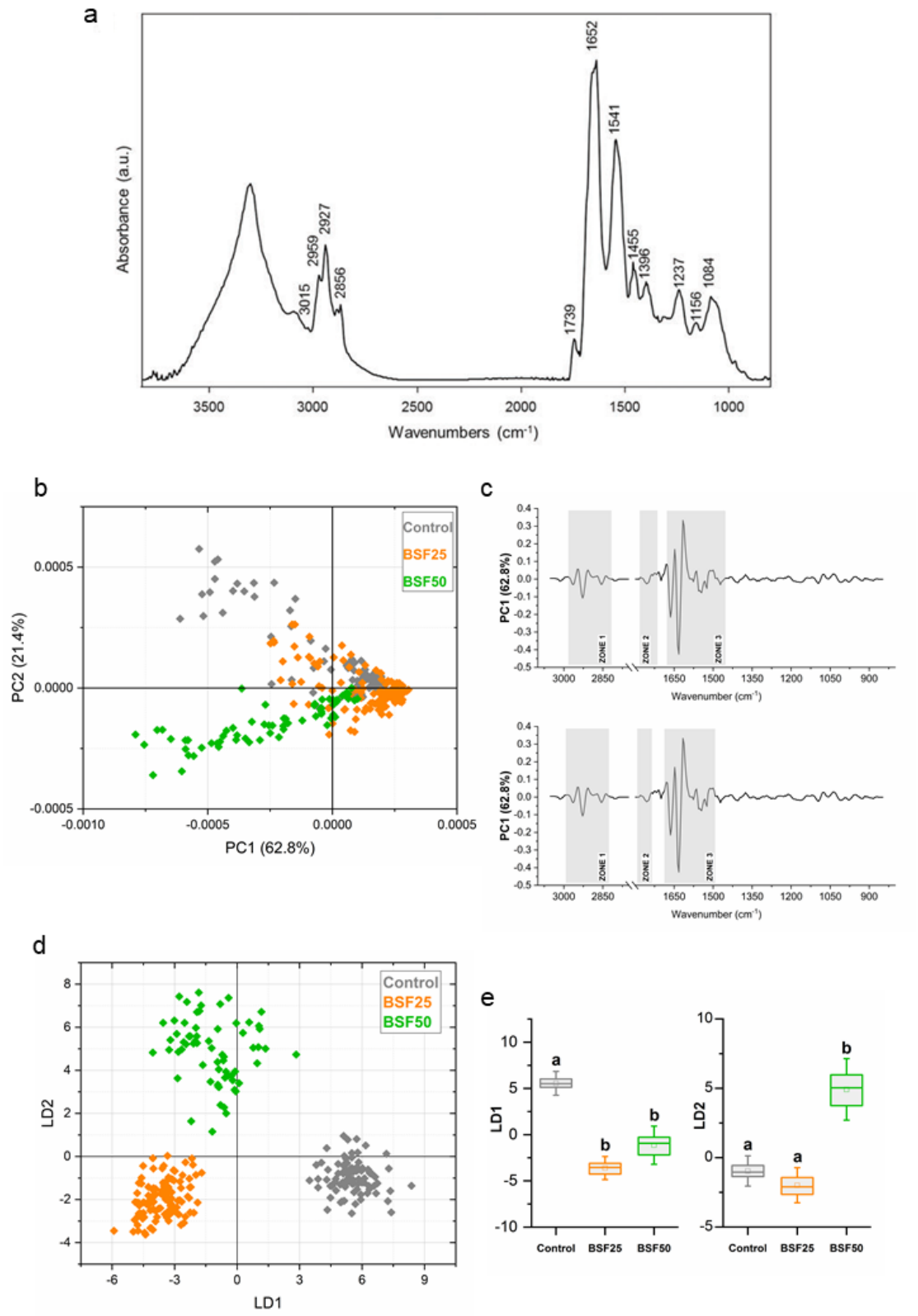

Figure 9. (**a**) Average IR spectrum of class IV oocyte from Control group. The IR spectrum is shown in absorbance mode in the 3800-800 cm^{-1} range. The wavenumbers (cm^{-1}) of the most relevant IR bands are reported. (**b**) PCA scores plot of Control, BSF25, and BSF50 second-derivative spectra. The percentages of the variance explained by PC1 and PC2 are reported in parentheses. (**c**) Loading spectra of PC1 and PC2. (**d**) PCA-LDA score plot of Control, BSF25, and BSF50 second-derivative spectra. LD1 and LD2 represent the first and second linear discriminant functions obtained by the canonical variable scores of PCA-LDA. (**e**) One-dimensional score plots of LD1 and LD2. Box chart legend: center line = median; center square = mean; edges = 25th and 75th percentile; whiskers = SD. Different letters over box charts indicate statistically significant differences among groups ($p < 0.05$). Zebrafish fed diet based on FM (Control) and diets with 25 (BSF25) or 50 % (BSF50) replacement of FM with full-fat BSF prepupae meal.

compounds); and PH-CARBO/CELL (relative amount of phosphates and carbohydrates). CELL, calculated as sum of the integrated areas of the regions 3000-2824 and 1780-950 cm^{-1}, was considered representative of the total cell biomass. With regard to lipids (LIP/CELL), a statistically significant, increase was found in BSF25 and BSF50 experimental groups; moreover, in BSF50, a significant increment in fatty acids and SFA (FA/CELL and CH2/CELL) and a significant decrement of unsaturated ones (CH/CELL) were detected. A significant decrease in protein amount (PRT/CELL,) and COO- groups of amino acids (COO/CELL) was observed only in BSF50. No significant changes were highlighted in phosphates (PH/CELL) and carbohydrates (PH-CARBO/CELL), while a significant decrement of glycosylated compounds (COH/CELL) was found in BSF50.

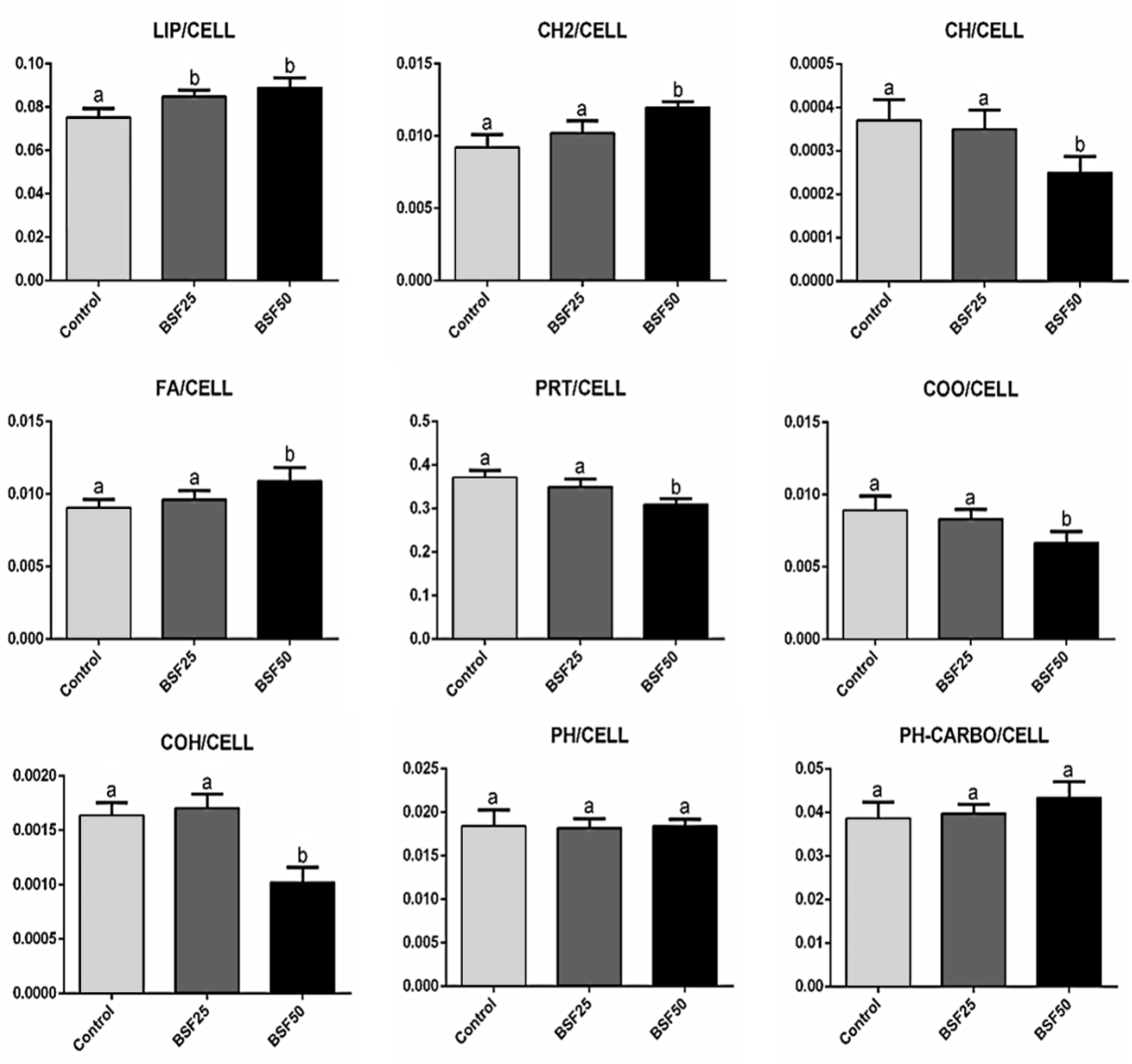

Figure 10. Biochemical composition of the inner compartment of class IV oocytes from Control, BSF25, and BSF50 groups. Statistical analysis of the numerical variation of the following band area ratios: LIP/CELL, CH2/CELL, CH/CELL, FA/CELL, PRT/CELL, COO/CELL, COH/CELL, PH/CELL, and PH-CARBO/CELL. Different letters indicate statistically significant differences among experimental groups. Values are presented as mean ± SD (n = 15). Zebrafish fed diet based on FM (Control) and diets with 25 (BSF25) or 50 % (BSF50) replacement of FM with full-fat BSF prepupae meal.

Real-time PCR results

Real time PCR analysis performed on genes involved in the long-chain PUFA biosynthesis is presented in Figure 11. Regarding *elovl2* gene expression, a dietary BSF meal dose-dependent increase in gene expression was observed. BSF50 group showed a significant upregulation compared to Control, while BSF25 did not show significant differences with respect to the other experimental groups. Considering results about *elovl5* gene expression, the BSF50 group showed a significantly higher value compared to BSF25, while Control and BSF25 did not show significant differences between them. Finally, *fads2* gene expression did not show significant differences among the experimental group.

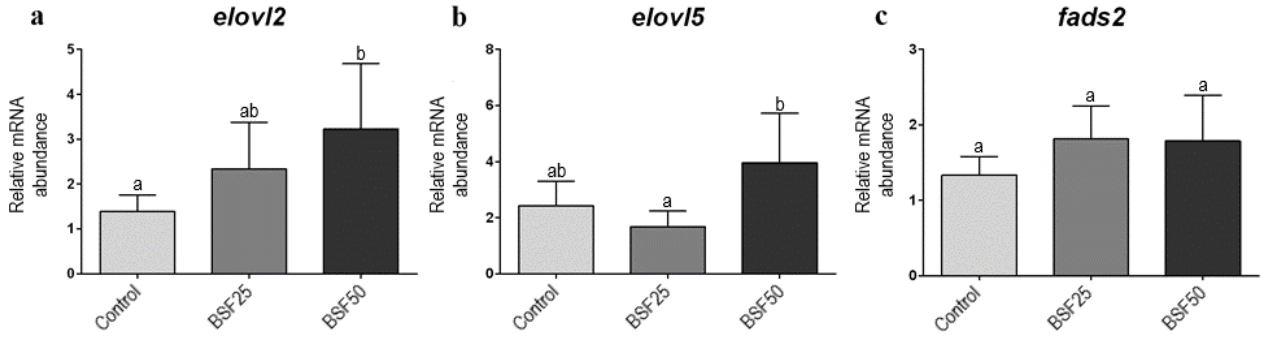

Figure 11. Relative mRNA levels of genes involved in long-chain PUFA biosynthesis analysed in liver samples from female zebrafish. (**a**) *elovl2*, (**b**) *elovl5*, and (**c**) *fads2*. Different letters indicate statistically significant differences among experimental groups ($p < 0.05$). Values are presented as mean ± SD (n = 5). Zebrafish fed diet based on FM (Control) and diets with 25 (BSF25) or 50 % (BSF50) replacement of FM with full-fat BSF prepupae meal.

Discussion

In this study, the inclusion of BSF meal in the diets provided interesting results on zebrafish reproductive pathways. As reported in several reviews and papers on the use of insect meal in aquafeed [**62**-**67**], insects are characterized by high quantity (60-80%) and quality of proteins and are rich in essential amino acids. On the other hand, insects show an unbalanced fatty acids profile [**68**,**69**], rich in SFA rather than in PUFA, and this is known to affect fish growth and welfare, especially when high inclusion levels are used [**70**-**72**]. Recently, BSF prepupae fatty acids composition has been modified by varying the growth substrate [**72**]. Specifically, it has been demonstrated that culturing BSF prepupae on an organic substrate enriched 10% *Schizochytrium* sp. significantly increased the PUFA content of insects and may represent a proper method for further studies [**30**]. It is well established that lipid nutrition is particularly important for fish [**60**,**73**], since deficiencies in some of these molecules, such as arachidonic acid (20:4n6; ARA), EPA, and DHA can cause a general decrease of fish health, poor growth, low feed efficiency, anaemia and high mortality [**74**]. Presently, most of the studies investigated the role of PUFA on fish body composition and fish physiological responses [**75**-**77**]; however, only few studies were focused on the effects of

these molecules on fish reproduction [**78**-**80**]. Modern investigations should take advantage of several available laboratory techniques, to have a comprehensive overview of fish responses. In this study, some classical techniques like biometry, histology, and gas chromatography were coupled with more innovative ones like molecular and spectroscopic analysis. Specifically, FTIR spectroscopy, a fast, new, and label-free inexpensive technique, can be considered a complement to the traditional histological analysis since it is important to obtain biochemical information on the composition of biological samples through the macromolecule structure identification (lipid, protein, carbohydrates, and nucleic acids).

Lipid, and specifically PUFA, are essential to properly sustain fish reproduction and have been shown to deeply influence gonadal development, gamete quality, spawning success, and embryos hatchability [**81**-**83**]. In particular, during gametogenesis, PUFA are mobilized from the storage sites (muscle and liver), transferred to the ovaries, incorporated in the egg as yolk, and used as main nutritional source by the embryo. In addition, neutral lipids are stored in the form of oil droplets, which increase in number as the oocyte increase in size [**84**].

In this study, it was observed that the inclusion of BSF prepupae in the diets caused a dietary reduction in PUFA and a parallel increase in SFA. This unbalanced dietary lipid profile did not affect fish growth (with the exception of a length reduction) and GSI, while deeply influenced fish fatty acids composition, which reflected the same trend of SFA and PUFA shown by the tested diets. The same result was not evidenced in the fatty acids composition of class IV oocytes and fertilized eggs. Specifically, similar amounts of SFA, MUFA, and PUFA were found in Control and BSF25 groups, while a higher SFA and lower PUFA content were detected in BSF50 by gas chromatography. FTIR analysis confirmed these lipid changes (LIP/CELL, CH2/CELL, CH/CELL, and FA/CELL) in class IV oocytes from BSF50 group. In particular, length and unsaturation rates of lipid aliphatic chains and the amount of fatty acids were consistent with the fatty acids content and composition revealed by gas chromatography.

Usually, in fish, the primary mechanism responsible for increased PUFA biosynthesis during limited dietary PUFA intake is through upregulated expression of desaturase and elongase mRNA [**85**] able to convert shorter-chain precursors (moved from storage sites like muscle and liver) in highly unsaturated fatty acids. Accordingly, in this study, fish fed BSF diets showed a higher liver gene expression of these specific genes. The differences in fatty acids composition between the fish carcasses and the spawned eggs are a very interesting result, which underlines a significant investment of female zebrafish in the reproductive event, strongly suggesting the selective accumulation of these fatty acids in fish eggs (as revealed by FTIR and gas chromatography analyses).

In many freshwater demersal spawners, eggs usually lack a prominent oil globule, and vitellogenin is thus the most important lipid carrier into growing oocytes [**86**]. In these fish, vitellogenin-derived yolk contains ~20% lipid by weight, and about 60-80% of these lipids are represented by phospholipids, which are typically rich in PUFA and are important membrane components for the developing embryo [**87**]. In contrast with the vitellogenin-associated lipids, oil globules mainly contain neutral lipids rich in MUFA which, in fish, preferentially serve as metabolic energy reserve [**88**]. Oil droplets are extremely abundant in pelagic spawners, like marine perciforms, and can occupy up to half or more of the ooplasm volume [**89**,**90**]. Because zebrafish is a demersal freshwater spawner, completely lacking oil globules, the accumulation of MUFA in this species is not a prominent process and this could explain why these fatty acids did not show significant differences among the experimental groups. However, histological analysis revealed a higher amount of lipid droplets in class IV oocytes from BSF50 group, confirming the results obtained by FTIRM which showed a higher amount of the total lipid fraction in this class.

In this study, it was evidenced that fish were perfectly able to cope with the lower (25%) dietary BSF prepupae meal inclusion level respect to FM, resulting in similar reproductive performances compared to Control. Conversely, the BSF50 group showed a general decrease in spawned eggs, possibly due to a delay in oocyte maturation (greater abundance of previtellogenic oocytes detected by histological analyses) caused by the necessity of a longer ex novo PUFA biosynthesis by the fish and subsequent oocyte accumulation, or by the activation of atretic processes. This last hypothesis is supported by the increase in atretic oocytes and by a POF reduction in BSF50 with respect to BSF25 and Control. This specific condition caused a general reduction in the number of spawned eggs by the BSF50 group but did not affect the hatching success of the same experimental group, which was comparable to that observed in Control and BSF25 ones. This is one more evidence of the extraordinary effort that female fish put in the reproductive event, choosing for quality rather than quantity by activating specific processes to select those oocytes that should be ovulated or reabsorbed.

In conclusion, the results obtained in this study showed that the dietary substitution of FM with full-fat BSF prepupae meal up to 25% did not affect reproductive performances of female zebrafish, while a higher substitution (50% with respect to FM) resulted in reproductive impairments, specifically in terms of number of spawned eggs. These results suggest that the application of new ingredients for aquafeed formulation should always be deeply investigated not only during the different life stages of fish but also during the reproductive event. Even if zebrafish, due to its high reproductive rate and to the abundant information that has recently become available from genomic sequencing, is one of the most studied experimental models in biomedical sciences, developmental biology, genetics, toxicology, and aquaculture, a standardized diet for this species is still lacking. A few specific

zebrafish diets are now available in the market. However, it is not clear how much information has been collected by these companies on the physiological effects on fish, a main critical bottleneck that still remains. Nowadays, a great variety of feeds are usually used by the different research facilities to feed their zebrafish, possibly posing some limits on an easy comparison of the obtained research results. It is thus clear how, a standardized diet, sustained by solid research on the physiological effects on fish, is now extremely necessary to the zebrafish community.

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Chapter 3

Effects of “enriched” full-fat BSF-based diets on zebrafish larval development, growth, welfare, and progeny

3.1

Black Soldier Fly (*Hermetia illucens*) reared on roasted coffee by-product and *Schizochytrium* sp. as a sustainable terrestrial ingredient for aquafeeds production

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Abstract

Promoting circularity in the aquaculture sector through the conversion of great amount of organic by-products produced on land is a valuable strategy for a further development of the aquaculture sector. In this regard, insects represent a very promising example of bio-converting organisms; their application in aquafeeds, however, still faces possible limitations because of their lack in polyunsaturated fatty acids and the presence of chitin.

The aim of the present study was to apply circularity to Black Soldier Fly (*Hermetia illucens*) culture and to improve the insects' biomass fatty acids composition by culturing them on a land-produced by-product (coffee silverskin) enriched with a 10% *Schizochytrium* sp. The insect biomass was then used to formulate five fish diets containing 0, 25, 50, 75 and 100 % of insect meal respect to fish meal, respectively. Diets were used for a feeding trial during zebrafish (*Danio rerio*) larval development (21 days) and a multidisciplinary approach including biometry, histology, gas chromatography, spectroscopy (FTIR), microbiota analyses and molecular biology was applied to better understand fish responses to the new diets. Results showed that the 50% substitution of fish meal with insect meal represented the best compromise between ingredient sustainability and proper fish growth and welfare. Fish fed with higher BSF inclusions (75 and 100 %) showed a severe degree of hepatic steatosis, microbiota modification, a higher lipid content (FTIR), fatty acid modification and higher expression of both stress and immune response markers.

Introduction

Aquaculture is the fastest growing food production sector worldwide, and it is estimated that by 2030 62% of food-fish will come from aquaculture [**1**]. The reason for this growth relies on several factors, including wild capture fisheries decline, increase in global demand for seafood products and the 9 billion people population expected on Earth by 2050, with the consequent doubling of farmed production required to meet the mid-century demand for seafood [**3**,**4**]. Because of the estimated increase in World's population, a significant rise in waste and by-products production is expected. The EC Directive No. 2008/98, which establishes the order of priority in the choice of by-products treatments (with their reuse as favoured option and their landfill disposal as last option), will play a central role for further development of a European circular economy. In consideration of this, aquaculture should be more responsible, sustainable, innovative, based on the circular economy concept, and able to provide larger volumes of healthy food by using environmentally friendly ingredients while promoting fish needs and welfare [**4-7**].

For many years aquaculture has relied on the use of fish meal (FM) and fish oil (FO) as main ingredients in aquafeeds [**8**,**9**]. For its further development, however, nutritious and sustainable

ingredients must be identified and tested [**10-12**]. Among such ingredients, several options have already been investigated and great attention has been addressed to animal by-products, vegetable sources and microalgae [**13-16**], but unfortunately each of them has some downsides in the application for aquafeed formulation [**17-21**].

Insects represent a very promising example of bio-converting organisms [**22-24**]. Most insect species are farmed on land-produced by-products [**25-28**] and, in addition, farming of insects is sustainable in terms of land use, water consumption and CO_2 production, because of their low environmental requirements [**29**,**30**]. Land organic by-products are thus efficiently bio-converted in a highly nutritious biomass which, in turn, can provide sustainable new ingredients for fish nutrition. Specifically, every year, up to 200˙000 tons of coffee silverskin are produced by the coffee industry as by-products [**31-32**]. Therefore, in a circular economy perspective, coffee silverskin may represent a suitable substrate for insect production. Among several insect species, the Black Soldier Fly (*Hermetia illucens*; BSF) has a promising role for aquafeed production. From a nutritional point of view, the BSF accumulates good amounts of proteins and lipids (307.5-588.0 g/kg and 113.0-386.0 g/kg, respectively [**33**,**34**]. However, while the essential amino acid composition is approximately similar to that of FM [**35**], the fatty acids profile is extremely different, posing some limits in the full-fat BSF meal inclusion in aquafeeds [**36**,**37**]. In terms of fatty acids profile, the BSF is usually rich in saturated (SFA) and poor in polyunsaturated (PUFA) ones [**22**], which are extremely important for fish [**38**]. PUFA deficiencies during fish farming can cause a general decrease of fish health, poor growth, low feed efficiency, anaemia and high mortality [**39-42**].

Because of their fatty acids profile, insects are mainly used as protein source in fish nutrition and often undergo a defatting process which represents an important cost in their manufacture [**43-45**]. Therefore, in aquafeed production it is desirable to use full-fat insect meal. In this regard, it is known that insects are able to modulate their fatty acids composition in relation to the growth substrate [**46**,**47**]. Recently, some authors demonstrated that rearing BSF larvae on an organic substrate containing proper amounts of omega-3 PUFA was a suitable procedure to improve the fatty acids profile of the final insect biomass [**48**,**49**].

New ingredients to be introduced in aquafeeds must be carefully analysed, since it is well established that different feed ingredients may have modulatory effects of on fish physiological responses and gut microbiota [**50**,**51**]. Besides zootechnical indexes, several molecular markers involved in fish growth, stress response, lipid metabolism, appetite and immune response [**37**, **52-54**] have been proposed as valid tools to precociously detect physiological responses in fish fed new diets and represent an up-to-date and important approach. In addition, it is already known that insects possess natural antibiotic properties, possibly modifying fish microflora [**55**,**56**], but knowledge concerning

interactions between insect-based diets, gut microbiota, and the aforementioned markers is still fragmentary and incomplete.

The aim of the present study was to interconnect land and aquatic environment by: (i) recycling land-produced organic by-products (coffee silverskin) to rear BSF larvae; (ii) enriching the insects growth substrate with *Schizochytrium* sp. to improve their fatty acids profile; (iii) producing highly nutritious full-fat BSF prepupae meal; (iv) testing the biological effects of diets including graded inclusions of BSF prepupae meal respect to FM in an aquatic experimental model organism, the zebrafish (*Danio rerio*) and (v) applying a multidisciplinary approach integrating biometric, histological, gas chromatographic, molecular, microbiological and spectroscopic analyses to better understand the physiological responses of the fish. Zebrafish represents an extraordinary experimental model for aquaculture, biomedical, developmental biology, genetics, toxicology studies, due to its high reproductive rate and abundant information that has recently become available from genome sequencing [**57**,**58**]. Particularly, zebrafish are used to generalize how several biological processes take place in related organisms (like finfish species) and contribute to understand the mechanisms involved in fish nutrition, welfare and growth, possibly providing useful information for finfish production [**59-62**].

Methods

Ethics

All procedures involving animals were conducted in line with the Italian legislation on experimental animals and were approved by the Ethics Committee of the Università Politecnica delle Marche (Ancona, Italy) and the Italian Ministry of Health (626/2018-PR). Optimal rearing conditions (see further section for details) were applied throughout the study, and all efforts were made to minimize animal suffering by using an anaesthetic (MS222; Merck KGaA, Darmstadt, Germany).

Insects feeding substrate preparation

The main component of the insects feeding substrate consisted of coffee silverskin, an industrial by-product provided by Saccaria Caffe' srl (Marina di Montemarciano, Ancona, Italy). Coffee by-product (moisture 44%) was collected and grinded in an Ariete 1769 food processor (De Longhi Appliances Srl, Italy) to a 2 ± 0.4 mm particle size before the feeding substrate preparation. The diet for the insects was formulated including 10% (w/w) of *Schizochytrium* sp. to the coffee by-product (for details, please see Truzzi et al. [**63**]. The freeze-dried *Schizochytrium* sp. was provided by AlghItaly Società Agricola srl (Sommacampagna, Verona, Italy). In the insects feeding substrate, distilled water was added to reach a final moisture of ~70% [**64**].

Insects rearing

Insects were reared in a climatic chamber at a 27 ± 1 °C temperature, relative humidity of 65 ± 5 % [**65**], in continuous darkness. Six days old larvae (purchased from Smart Bugs s.s. Ponzano Veneto, Treviso, Italy) were hand-counted and divided in groups of 640 larvae per replicate (n = 65) for a total of 41,600 specimens. Each replicate consisted of a plastic box (57 × 38 × 16 cm) screened with fine-mesh cotton gauze and covered with a lid with 90 ventilation holes of 0.05 cm diameter [**65**]. Larvae were reared at a density of 0.3/cm^2 [**66**], with a feeding rate of 100 mg/day [**67**] that was prepared and added once a week (448 g for each box). Insects were visually inspected every day and, when prepupae were identified by the change in tegument colour from white to black [**68**], they were collected, washed, dried and stored at -80 °C.

Fish diets production

Full-fat BSF prepupae were freeze dried, grinded with Retsch Centrifugal Grinding Mill ZM 1000 (Retsch GmbH, Haan, Germany) and used to prepare the experimental diets. A control diet (Hi0) containing FM, wheat gluten, pea protein concentrates and FO as major ingredients, was prepared according to a commercially available standard diet for zebrafish (Zebrafeed, Sparos ltd, Portugal). The experimental diets were iso-nitrogenous (50%) and iso-lipidic (13%). Insect-based diets were prepared by including graded levels of full-fat BSF prepupae meal respect to FM (25, 50, 75, and 100 %, referred as Hi25, Hi50, Hi75 and Hi100, respectively) in the Hi0 formulation. The conventional vegetable ingredients (pea protein concentrate and wheat gluten) used to formulate the experimental diets were maintained approximatively at constant (0.7:1 w:w) ratio in all the diets. In summary, all the grounded ingredients (0.5 mm) and FO were thoroughly blended (Kenwood kMix KMX53 stand Mixer; Kenwood, De Longhi S.p.a., Treviso, Italy) for 20 min and then water was added to the mixture to obtain an appropriate consistency for pelleting. Pellets were obtained by using a 1 mm die meat grinder and dried at 40 °C for 48-72 h. The obtained diets were then grinded and stored in vacuum bags at -20 °C until use. Feed samples were analysed for moisture (AOAC #950.46), crude protein (AOAC #976.05), ash (AOAC #920.153) and ether extract (AOAC #991.36) contents according to AOAC [**69**]. The total lipid fraction of the test diets was extracted using chloroform-methanol (2:1 v:v) (Merck KGaA) mixture according to Folch et al. [**70**]. Diet formulation and proximate composition are shown in Table 1.

Fish

Zebrafish AB embryos were maintained for 48 h in a Tecniplast system (Varese, Italy) in the following conditions: 28 °C temperature, pH 7.0, NH_3 and NO_2^- concentrations < 0.01 mg/L, NO_3^-

concentration < 10 mg/L, and photoperiod 12L/12D, respectively [**71**]. After this period, embryos were gently collected, counted under a stereomicroscope and randomly divided in five experimental groups (in triplicate) according to the five test diets.

Table 1. Ingredients (g/kg) and proximate composition (g/100 g) of the experimental diets used in the present study.

	Hi0	**Hi25**	**Hi50**	**Hi75**	**Hi100**
Ingredients (g/kg)					
Fish meal [1]	470	400	250	110	-
Vegetable mix [2]	220	230	298	385	440
BSF prepupae meal	-	115	235	350	460
Wheat flour [3]	198	172	120	110	72
Fish oil	80	51	25	10	-
Soy lecithin	8	8	8	11	4
Mineral and Vitamin supplements [4]	14	14	14	14	14
Binder	10	10	10	10	10
Proximate composition (%)					
Dry Matter	97.1 ± 0.1	95.8 ± 0.1	94.9 ± 0.1	93.6 ± 0.1	92.7 ± 0.1
Crude protein	51.6 ± 0.1	50.7 ± 2.6	50.4 ± 0.3	51.2 ± 1.5	50.5 ± 3.1
Ether extract	14.4 ± 0.6	13.1 ± 0.4	12.9 ± 0.4	13.2 ± 0.5	13.0 ± 0.5
Nitrogen-free extract	21.3 ± 0.3	20.8 ± 1.0	20.6 ± 0.5	19.0 ± 0.7	18.5 ± 1.3
Ash	9.8 ± 0.2	11.1± 0.01	11.0 ± 0.0	10.1 ± 0.1	10.7 ± 0.1

[1] Raw ingredient kindly supplied by Skretting Italia. **[2]** Vegetable mix (pea protein concentrate: wheat gluten, 0.7:1 w/w) Lombarda trading srl, Casalbuttano & Uniti (Cremona, Italy) and Sacchetto spa (Torino, Italy). **[3]** Consorzio Agrario (Pordenone, Italy). **[4]** Mineral and Vitamin supplement composition (% mix): $CaHPO_4.2H_2O$, 78.9; MgO, 2.725; KCl, 0.005; NaCl, 17.65; $FeCO_3$, 0.335; $ZnSO_4.H_2O$, 0.197; $MnSO_4.H_2O$, 0.094; $CuSO_4.5H_2O$, 0.027; Na_2SeO_3, 0.067; thiamine hydrochloride (vitamin B1), 0.16; riboflavin (vitamin B2), 0.39; pyridoxine hydrocloride (vitamin B6), 0.21; cyanocobalamine (vitamin B12), 0.21; niacin (vitamin PP or B3), 2.12; calcium pantotenate, 0.63; folic acid, 0.10; biotin (vitamin H), 1.05; myo-inositol (vitamin B7), 3.15; stay C Roche (vitamin C), 4.51; tocopherol (vitamin E), 3.15; menadione (vitamin K3), 0.24; retinol (vitamin A 2500 UI/ kg diet), 0.026; cholecalciferol (vitamin D3 2400 UI/kg diet), 0.05; choline chloride, 83.99. For proximate composition, values are reported as mean of triplicate analyses.

Experimental design

Zebrafish larvae were maintained in fifteen 20 L tanks to set up the five experimental dietary treatments; each experimental group was composed of 1500 larvae (500 larvae per tank). The water in the larval tanks had the same chemical-physical characteristics of the broodstocks' tank and was gently replaced 10 times a day by a dripping system [**72**]. The sides of each tank were covered with black panels to reduce light reflection. All tanks were siphoned 30 min after feeding (twice a day) to remove possible feed excess and dead larvae. The required larvae were sampled 20 days after fertilization (dpf), euthanized with a lethal dose of MS222 (1 g/L, Merck KGaA) and properly stored for further analyses.

Feeding schedule

Starting from 5 dpf to 20 dpf, zebrafish larvae were fed as follows: (i) Hi0 (control) group: larvae fed the diet including 0% of full-fat BSF prepupae meal (Hi0 diet); (ii) Hi25, Hi50, Hi75, Hi100 groups: larvae fed the diet including 25, 50, 75, 100 % respectively of full-fat BSF prepupae meal respect to FM. Zebrafish larvae were fed on the experimental diets (2% body weight; 100-250 µm size) twice a day and, in addition, from 5 to 10 dpf, all groups were fed (one feeding in the morning) on the rotifer *Brachionus plicatilis* (5 ind/mL) according to Lawrence et al. [**73**] and Piccinetti et al. [**74**].

Biometry

Five zebrafish larvae (15 per dietary group) were randomly collected from the different tanks of each experimental group at hatching (3 dpf) and at the end of the experiment (20 dpf). The standard length was determined using a sliding calliper (precision: 0.1 mm) and the wet weight using an analytical balance (precision: 0.1 mg). At 3 dpf, wet weight was measured on pools of five larvae in triplicate. For each experimental group, specific growth rate (SGR) was calculated as follows: SGR% = [(lnWf - lnWi)/t] x 100, where Wf is the final wet weight, Wi, the initial wet weight, and t, the number of days (17). Survival rate in all experimental groups was about 85%.

Fatty acids composition

Experimental diets (three samples per diet) and fish larvae samples (four pools of zebrafish larvae of 1.5 g each per tank; 12 for dietary group) were analysed for fatty acids composition. Samples were minced and homogenized (homogenizer MZ 4110, DCG Eltronic, Monza, Italy), and larvae were also freeze-dried (Edwards EF4, Crawley, Sussex, England). Aliquots of 200 mg of each sample were added with 100 µl of Internal Standard (methyl ester of nonadecanoic acid, 99.6%, Dr. Ehrenstorfer GmbH, Augsburg, Germany), and extracted overnight following the method of Folch et al. [**70**]. Analyses were carried out on three aliquots per sample. All lipid extracts were evaporated under laminar flow inert gas (N_2) until constant weight and re-suspended in 0.5 ml of n-epthane. Fatty acids methyl esters (FAME) were prepared according to Canonico et al. [**75**] using methyl ester of nonadecanoic acid (19:0; Dr. Ehrenstorfer GmbH) as internal standard. FAME were determined using an Agilent-6890 GC System (Milano, Italy) coupled to an Agilent-5973 N quadrupole Mass Selective Detector (MSD) (Milano, Italy). A CPS ANALITICA CC-wax-MS (30 m × 0.25 mm ID, 0.25 µm film thickness) capillary column was used to separate FAME. Instrumental conditions for the studied matrices were set up according to Truzzi et al. [**76**,**77**]. For each analysed aliquot of sample, at least three runs were performed on the GCMS.

Histology

Nine zebrafish larvae per dietary group (3 larvae per tank, at 20 dpf), randomly collected from the different tanks, were fixed by immersion in Bouin's solution (Merck KGaA) and then stored at 4 °C for 24 h. Larvae were washed three times in ethanol (70%) for 10 min and preserved in the same ethanol solution. Larvae were then dehydrated in graded ethanol solutions (80, 95 and 100 %), washed in xylene (Bio-Optica, Milano, Italy) and embedded in paraffin (Bio-Optica). Solidified paraffin blocks were cut with a microtome (Leica RM2125 RTS, Nussloch, Germany) and 5 μm sections were stained with Mayer hematoxylin and eosin Y (Merck KGaA). Sections were observed using a Zeiss Axio Imager.A2 (Zeiss, Oberkochen, Germany) microscope in order to study the hepatic parenchyma and intestine morphology. Images were acquired by mean of a combined colour digital camera Axiocam 503 (Zeiss). Moreover, to ascertain the extent of fat accumulation in liver, a quantitative analysis was performed on a significant number of histological sections from each larva (3 larvae per tank, n = 9). Non-evaluable areas, such as blood vessels were not considered. The percentage of fat fraction (PFF) on the total tissue areas was calculated using the ImageJ software setting a homogeneous threshold value.

FTIR analysis

Fifteen zebrafish larvae per dietary group (5 per tank) were randomly collected at 20 dpf. Samples were minced, homogenized (homogenizer MZ 4110, DCG Eltronic) and freeze-dried (Edwards EF4) for FTIR analysis. For each group, five aliquots of 5 mg each were analysed. FTIR analysis was performed using a Spectrum GX1 Spectrometer equipped with a U-ATR accessory and a diamond/SeZn crystal (PerkinElmer, Waltham, Massachusetts, USA). Measurements were carried out in reflectance in the MIR region from 4000 to 800 cm^{-1} (spectral resolution 4 cm^{-1}). Each spectrum was the result of 64 scans. Before each sample acquisition, a background spectrum was collected on the clean surface of the crystal. Raw IR spectra were converted in absorbance, two-points baseline linear fitted in the 4000-800 cm^{-1} spectral range and vector normalized in the same interval (OPUS 7.1 software package).

For all experimental groups, the average absorbance spectra were calculated together with their standard deviation spectra (average absorbance spectrum ± standard deviation spectra) and analysed to identify the most featuring IR peaks (in terms of position/wavenumbers). Then, average absorbance spectra and their standard deviation spectra were curve-fitted in the 3050-2800 cm^{-1} and 1790-900 cm^{-1} regions upon two-points baseline correction and vector normalization. A Gaussian algorithm was adopted, and the number and position of the underlying peaks was defined by second derivative analysis of the spectra (GRAMS/AI 9.1, Galactic Industries, Inc., Salem, NH). In the 3050-2800 cm^{-1}

region, the following underlying peaks were identified: ~3010 cm^{-1} (=CH moieties in unsaturated lipid alkyl chains); ~2959 cm^{-1} and ~2872 cm^{-1} (CH3 groups in lipid alkyl chains); ~2925 cm^{-1} and ~2854 cm^{-1} (CH2 groups in lipid alkyl chains). In the 1790-900 cm^{-1} region, the following underlying peaks were identified: ~1744 cm^{-1} (C=O moiety in lipids and fatty acids); ~1639 and ~1536 cm^{-1} (respectively Amide I and II bands of proteins); ~1457 cm^{-1} (proteins side chains); ~1390 cm^{-1} (COO^- groups in aspartate and glutamate amino acids); ~1234 cm^{-1} (collagen); ~1157 cm^{-1} (glycosylated compounds); ~1080 cm^{-1} (phosphate groups), and ~1055 cm^{-1} (mucin). The integrated areas of the most relevant peaks were calculated and used to evaluate the following band area ratios: LIP/TBM (representing the overall amount of lipids), 1744/LIP (representing the amount of fatty acids compared to lipids), 2928/LIP (representing the amount of SFA with respect to lipids), and 3010/LIP (representing the amount of unsaturated fatty acids compared to lipids); PRT/TBM (representing the overall amount of proteins), 1234/PRT (representing the amount of collagen compared to proteins), and 1055/PRT (representing the amount of mucin compared to proteins). TBM was the sum of the integrated areas of all peaks in the 3050-2800 cm^{-1} and 1790-900 cm^{-1} regions; LIP was the sum of the integrated areas of all peaks in the 3050-2800 cm^{-1} region, while PRT was the sum of the integrated areas of the bands at 1639 and 1536 cm^{-1}.

Microbiome

RNA extraction and cDNA synthesis. Prior to analysis, zebrafish larvae (60 larvae per dietary group) were disinfected by washing in 50 mL of ethanol (70%) on a laboratory shaker (VDRL Stirrer with thermostatic cupola, ASAL s.r.l, Milan, Italy) at 150 rpm for 1 min at room temperature, in order to analyse only the gut microbiome. These samples were thus identified as G (gut) Hi0, Hi25, Hi50, Hi75 and Hi100 for these analyses. After discharging the ethanol, the samples were rinsed in two additional washing steps in 50 mL of sterile deionized water. Subsequently, 31.5 mL of sterile peptone water (peptone, 1 g/L) was added to each sample, which was then homogenized in a Stomacher apparatus (400 Circulator, International PBI, Milan, Italy) for 3 min at 260 rpm. Then, 1.5 mL of each tenfold diluted (10-1 dilution) homogenate were centrifuged at 14˙000 rpm for 10 min, the supernatants were discarded, the obtained cell pellets covered with RNA later Stabilization Solution (Ambion, Foster City, CA, USA) and stored at -80 °C until use. The Quick-RNA Fungal/Bacterial Microprep kit (Zymo Research, CA, USA) was used for the extraction of total microbial RNA from the cell pellets following the manufacturer's instructions.

The extracted RNAs were checked for quantity, purity and integrity as previously described by Garofalo et al. [**78**]. Moreover, the extracts were amplified using the PCR universal prokaryotic primers 27f and 1495r [**79**] to exclude the presence of bacterial DNA contamination. Five µL of each

RNA sample were reverse-transcribed in cDNA using the SensiFAST cDNA Synthesis Kit for RT-qPCR (Bioline, London, UK) following the manufacturer's instructions.

16S rRNA amplicon target sequencing. cDNA was used as template in the PCR amplifying the V3-V4 region of the 16S rRNA gene using the primers and protocols described by Klindworth et al. [**80**]. PCR amplicons were cleaned using the Agencourt AMPure kit (Beckman Coulter, Milan, Italy) and tagged using the Nextera XT Index Kit (Illumina Inc. San Diego, CA) according to the manufacturer's instructions. After the 2nd clean-up step, amplicons were quantified using a QUBIT dsDNA Assay kit and an equimolar amount of amplicons from different samples were pooled. The library was denatured with 0.2 N NaOH, diluted to 12 pM, and combined with 20% (vol/vol) denatured 12 pM PhiX, prepared according to Illumina guidelines. The sequencing was performed with a MiSeq Illumina instrument (Illumina) with V3 chemistry and generated 250 bp paired-end reads according to the manufacturer's instructions.

Molecular analyses

RNA extraction and cDNA synthesis. Total RNA extraction from 5 zebrafish larvae from each tank (15 larvae per dietary group) was optimized using the RNAzol RT reagent (Merck KGaA) according to Piccinetti et al. [**81**]. The total RNA extracted was eluted in 40 µl of RNase-free water (Qiagen). The final RNA concentration was determined using a NanoPhotometer P-Class (Implen, München, Germany). RNA integrity was verified by GelRed™ staining of 28S and 18S ribosomal RNA bands on 1% agarose gel. RNA was stored at -80 °C until use. Finally, 2 µg of RNA were used for cDNA synthesis, using the High Capacity cDNA Reverse Transcription Kit (Bio-Rad, Milan, Italy) following the manufacturer's instructions.

Real-Time PCR. PCRs were performed with SYBER green method in an iQ5 iCycler thermal cycler (Bio-Rad) following Vargas et al. [**12**]. Relative quantification of the expression of genes involved in fish growth (insulin-like growth factor 1, *igf1*; insulin-like growth factor 2a, *igf2a;* myostatin, *mstnb*), stress response (glucocorticoid receptor, *nr3c1*; heat shock protein 70, *hsp70.1*), long-chain PUFA biosynthesis (fatty acid elongase 2, *elovl2*; fatty acid elongase 5, *elovl5;* fatty acid desaturase 2, *fads2*), appetite response (ghrelin, *ghrl*; neuropeptide Y, *npy*; cannabinoid receptor 1, *cnr1*; leptin a, *lepa*), immune response (interleukin 1β, *il1b*; interleukin 10, *il10*; tumor necrosis factor a, *tnfa*) and enzymatic hydrolysis of chitin (chitinase 2, *chia.2*; chitinase 3, *chia.3*) was performed. Actin-related protein 2/3 complex, subunit 1A (*arpc1a*) and ribosomal protein, large, 13 (*rpl13*) were used as internal standards in each sample in order to standardize the results by eliminating variation in mRNA and cDNA quantity and quality. No amplification products were observed in negative controls and no primer-dimer formations were observed in the control templates. Amplification products were

sequenced, and homology was verified. The data obtained were analysed using the iQ5 optical system software version 2.0 (Bio-Rad) including GeneEx Macro iQ5 Conversion and genex Macro iQ5 files. Primer sequences were designed using Primer3 (210 v. 0.4.0) starting from zebrafish sequences available in ZFIN Primer sequences used were reported in Table 2.

Table 2. Sequences, Zebrafish Information Network (ZFIN) IDs, and annealing temperature (A.T.) of the primers used for the present study.

Gene	Forward Primer (5'-3')	Reverse Primer (5'-3')	ZFIN IDs	A. T. (°C)
igf1	GGCAAATCTCCACGATCTCTAC	CGGTTTCTCTTGTCTCTCTCAG	ZDB-GENE-010607-2	53
igf2a	GAGTCCCATCCATTCTGTTG	GTGGATTGGGGTTTGATGTG	ZDB-GENE-991111-3	59
mstnb	GGACTGGACTGCGATGAG	GATGGGTGTGGGGATACTTC	ZDB-GENE-990415-165	58
nr3c1	AGACCTTGGTCCCCTTCACT	CGCCTTTAATCATGGGAGAA	ZDB-GENE-050522-503	58
hsp70.1	TGTTCAGTTCTCTGCCGTTG	AAAGCACTGAGGGACGCTAA	ZDB-GENE-990415-91	58
elovl2	CACTGGACGAAGTTGGTGAA	GTTGAGGACACACCACCAGA	ZDB-GENE-011212-1	60
elovl5	TGGATGGGACCGAAATACAT	GTCTCCTCCACTGTGGGTGT	ZDB-GENE-040407-2	60
fads2	CATCACGCTAAACCCAACA	GGGAGGACCAATGAAGAAGA	ZDB-GENE-011212-1	60
ghrl	CAGCATGTTTCTGCTCCTGTG	TCTTCTGCCCACTCTTGGTG	ZDB-GENE-070622-2	58
npy	GTCTGCTTGGGGACTCTCAC	CGGGACTCTGTTTCACCAAT	ZDB-GENE-980526-438	60
cnr1	AGCAAAAGGAGCAACAGGCA	GTTGGTCTGGTACTTTCACTTGAC	ZDB-GENE-040312-3	60
lepa	CTCCAGTGACGAAGGCAACTT	GGGAAGGAGCCGGAAATGT	ZDB-GENE-081001-1	58
il1b	GCTGGGGATGTGGACTTC	GTGGATTGGGGTTTGATGTG	ZDB-GENE-040702-2	54
il10	ATTTGTGGAGGGCTTTCCTT	AGAGCTGTTGGCAGAATGGT	ZDB-GENE-051111-1	56
tnfa	TTGTGGTGGGGTTTGATG	TTGGGGCATTTTATTTTGTAAG	ZDB-GENE-050317-1	53
chia.2	GGTGCTCTGCCACCTTGCCTT	GGCATGGTTGATCATGGCGAAAGC	ZDB-GENE-040426-2014	64
chia.3	TCGACCCTTACCTTTGCACACACCT	ACACCATGATGGAGAACTGTGCCGA	ZDB-GENE-040426-2891	65
arpc1a	CTGAACATCTCGCCCTTCTC	TAGCCGATCTGCAGACACAC	ZDB-GENE-040116-1	60
rpl13	TCTGGAGGACTGTAAGAGGTATGC	AGACGCACAATCTTGAGAGCAG	ZDB-GENE-031007-1	59

Statistical analyses

All data (except for microbiome) were analysed by one-way ANOVA, with diet as the explanatory variable. All ANOVA tests were followed by Tukey's post-hoc tests. The statistical software package Prism5 (GraphPad Software) was used. Significance was set at $p < 0.05$ and all results are presented as mean ± SD.

For microbiome analyses, paired-end reads were first merged using the FLASH software [**82**]. Joint reads were quality filtered (at Phred < Q20) by QIIME 1.9.0 software [**83**] and the pipeline recently described [**84**]. Briefly, the USEARCH software version 8.1 (Edgar et al., 2011) was used for chimera filtering and clean sequences were clustered into Operational Taxonomic Units (OTUs) at 97% of similarity by UCLUST algorithms. Centroids sequences of each cluster were used for taxonomic assignment using the Greengenes 16S rRNA gene database. OTU tables were rarefied at 44412 sequences. The OTU table displays the higher taxonomy resolution that was reached.

Results

Biometry

The increasing inclusion levels of full-fat BSF prepupae meal resulted in a significant increase in the larval specific growth rate. In particular, no significant differences were detected between Hi0 (25.4 ± 0.7 %) and Hi25 (25.7 ± 1.0 %) groups, while Hi50, Hi75 and Hi100 groups (27.6 ± 0.5, 27.8 ± 0.4, 28.4 ± 0.3 %, respectively) showed significantly higher values compared to both Hi0 and Hi25 ones (Fig. 1).

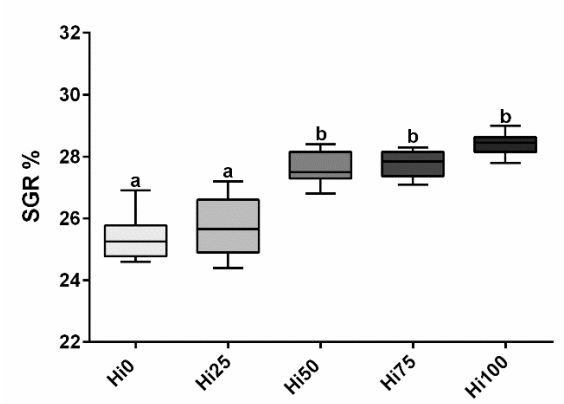

Figure 1. Specific Growth Rate (% weight growth/day) of zebrafish larvae. Boxplots show minimum and maximum (whiskers), first quartile, median and third quartile (box). Different letters denote statistically significant differences among experimental groups ($p < 0.05$). Zebrafish larvae fed diets including 0, 25, 50, 75 and 100 % of BSF prepupae meal respect to FM (Hi0, Hi25, Hi50, Hi75 and Hi100, respectively).

Fatty acids content and composition

Diets. The fatty acids classes percentages of the five experimental diets are presented in Figure 2a. BSF-based diets showed significantly higher percentages of SFA (40.9 ± 0.7, 40.0 ± 2.0, 35.9 ± 0.7 and 37.6 ± 2.8 % for Hi25, Hi50, Hi75 and Hi100 diets, respectively) compared to Hi0 diet (27.8 ± 1.3 %). All BSF-based diets showed significantly lower percentages of mono-unsaturated fatty acids (MUFA) (19.8 ± 0.3, 19.0 ± 0.9, 21.5 ± 0.2 and 20.0 ± 1.0 % for Hi25, Hi50, Hi75 and Hi100, respectively) and PUFA (39.3 ± 1.0, 41.0 ± 1.0, 42.6 ± 0.3 and 42.2 ± 3.2 % for Hi25, Hi50, Hi75 and Hi100, respectively) compared to Hi0 diet (24.7 ± 0.6 and 47.4 ± 1.4 % for MUFA and PUFA, respectively). In addition, increasing dietary inclusion levels of full-fat BSF prepupae meal resulted in a significant decrease of n3 percentages (from 38.8 ± 1.4 % for Hi0 diet to 11.1 ± 3.1 % for Hi100 diet) and a parallel significant increase in n6 percentages (from 8.6 ± 0.1 for Hi0 diet to 31.3 ± 0.9 % for Hi100 diet). Consequently, the n6/n3 ratio (Fig. 2b) showed significant differences among experimental diets, increasing from Hi0 (0.22 ± 0.01) to Hi100 (2.8 ± 0.2) diets. Finally, the higher was the BSF prepupae meal dietary inclusion, the higher was the n9 content (10.7 ± 0.2, 12.1 ± 0.7,

14.6 ± 0.2 and 15.2 ± 0.7 % for Hi25, Hi50, Hi75 and Hi100, respectively; Fig. 2a). The Hi0 diet showed an intermediate n9 content (13.9 ± 0.3 %) between Hi50 and Hi75 diets.

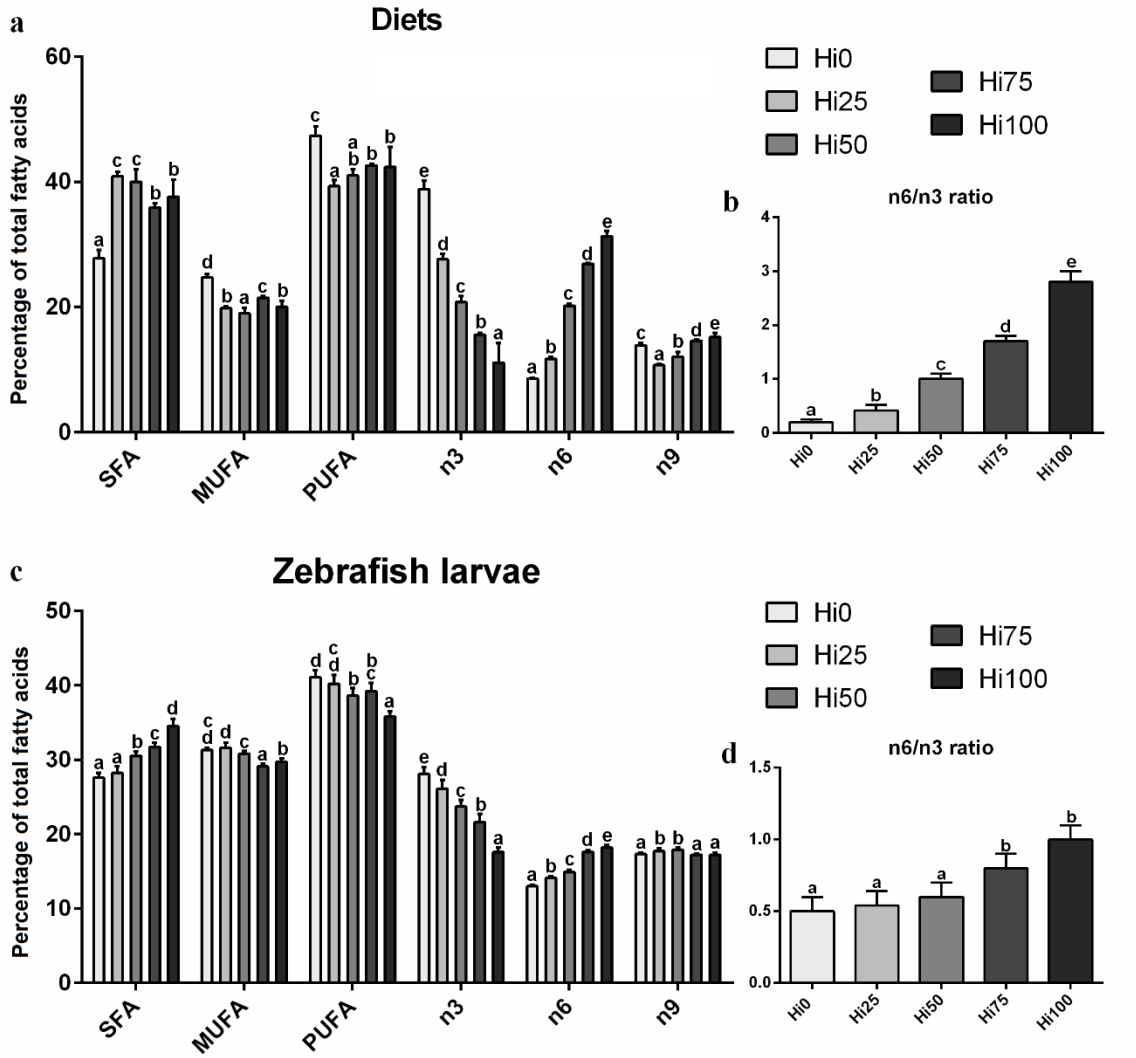

Figure 2. Content of SFA, MUFA and PUFA (as % of total fatty acids) and contribution of omega 3 (n3), omega 6 (n6) and omega 9 (n9) fatty acids to lipid profile. (**a**,**b**) experimental diets; (**c**,**d**) zebrafish larvae. Different letters indicate statistically significant differences among experimental groups compared within the same fatty acids class ($p < 0.05$). Values are presented as mean ± SD (n = 3 for experimental diets; n = 12 for zebrafish larvae). Zebrafish larvae fed diets including 0, 25, 50, 75 and 100 % of BSF prepupae meal respect to FM (Hi0, Hi25, Hi50, Hi75 and Hi100, respectively).

Zebrafish larvae. Figure 2c illustrates the fatty acids classes percentages of zebrafish larvae fed the different diets. The fatty acids profile of zebrafish larvae was deeply influenced by the BSF prepupae meal dietary inclusion. In particular, SFA percentage significantly increased with the inclusion of BSF prepupae meal in the diets (27.6 ± 0.6, 28.2 ± 0.9, 30.5 ± 0.6, 31.7 ± 0.6 and 34.5 ± 1.0 % for Hi0, Hi25, Hi50, Hi75 and Hi100, respectively), while both MUFA (31.3 ± 0.3, 31.6 ± 0.7, 30.8 ± 0.4, 29.1 ± 0.4 and 29.7 ± 0.5 % for Hi0, Hi25, Hi50, Hi75 and Hi100, respectively) and PUFA (41.1 ± 0.9, 40.2 ± 1.2, 38.6 ± 1.0, 39.2 ± 1.1 and 35.8 ± 0.7 % for Hi0, Hi25, Hi50, Hi75 and Hi100,

respectively) significantly decreased with the increasing dietary BSF prepupae meal inclusion levels respect to FM.

Table 3. Fatty acids composition (as % of total fatty acids) of zebrafish larvae fed the experimental diets.

	Hi0	Hi25	Hi50	Hi75	Hi100
10:0	0.02±0.01	0.05±0.01	0.10±0.01	0.08±0.01	0.09±0.02
12:0	0.20±0.03 ^{a}	2.6±0.1 ^{b}	4.5±0.1 ^{c}	4.7±0.4 ^{c}	5.7±0.4 ^{d}
13:0	0.06±0.01	0.06±0.01	0.07±0.01	0.06±0.01	0.07±0.01
14:0	4.0±0.1 ^{ab}	4.0±0.1 ^{a}	4.2±0.1 ^{b}	3.9±0.2 ^{a}	4.3±0.2 ^{b}
15:0	0.86±0.04	0.80±0.03	0.82±0.01	0.79±0.02	0.84±0.04
16:0	14.7±1.0 ^{c}	14.0±0.7 ^{ab}	13.8±0.5 ^{a}	14.6±0.1 ^{bc}	15.5±0.9 ^{d}
16:1n9	1.2±0.1 ^{a}	1.3±0.1 ^{b}	1.3±0.1 ^{c}	1.5±0.1 ^{d}	1.5±0.1 ^{d}
16:1n7	8.7±0.6 ^{c}	9.0±0.3 ^{c}	8.3±0.1 ^{b}	7.6±0.4 ^{a}	7.9±0.1 ^{a}
17:0	1.2±0.1 ^{b}	1.1±0.1 ^{a}	1.1±0.1 ^{a}	1.1±0.1 ^{a}	1.2±0.1 ^{b}
18:0	6.3±0.2 ^{c}	5.5±0.1 ^{a}	5.8±0.2 ^{b}	6.3±0.4 ^{c}	6.7±0.4 ^{d}
18:1n9	14.7±0.1 ^{a}	15.3±0.3 ^{b}	15.7±0.7 ^{c}	15.1±0.3 ^{b}	15.3±0.1 ^{b}
18:1n7	5.2±0.1 ^{d}	4.9±0.2 ^{c}	4.6±0.2 ^{b}	4.3±0.1 ^{a}	4.5±0.2 ^{b}
18:2n6	9.6±0.1 ^{a}	10.6±0.2 ^{b}	11.0±0.2 ^{c}	12.6±0.3 ^{d}	12.7±0.7 ^{d}
18:3n6	0.43±0.01	0.41±0.02	0.50±0.01	0.47±0.03	0.53±0.03
18:3n3	3.7±0.1 ^{c}	3.3±0.2 ^{b}	3.5±0.2 ^{c}	2.7±0.2 ^{a}	2.8±0.3 ^{a}
20:0	0.22±0.01	0.18±0.01	0.19±0.01	0.23±0.02	0.21±0.01
20:1n9	0.90±0.05	0.75±0.01	0.63±0.02	0.48±0.02	0.39±0.02
20:2n6	0.29±0.02	0.29±0.01	0.31±0.01	0.35±0.02	0.38±0.02
20:3n6	0.46±0.04 ^{a}	0.52±0.02 ^{b}	0.70±0.04 ^{c}	0.98±0.06 ^{d}	1.1±0.1 ^{e}
20:4n6	2.3±0.2 ^{a}	2.3±0.1 ^{a}	2.4±0.1 ^{b}	3.2±0.3 ^{c}	3.5±0.1 ^{d}
20:3n3	0.21±0.03	0.19±0.01	0.21±0.01	0.17±0.01	0.17±0.02
20:5n3	10.2±0.2 ^{e}	8.7±0.7 ^{d}	7.2±0.3 ^{c}	4.6±0.1 ^{b}	3.5±0.2 ^{a}
22:1n9	0.31±0.02	0.28±0.01	0.18±0.02	0.12±0.01	0.04±0.01
22:6n3	14.0±1.3 ^{c}	13.9±1.2 ^{c}	12.9±0.8 ^{b}	14.0±1.3 ^{c}	11.2±1.0 ^{a}
24:1n9	0.18±0.02	0.09±0.01	0.08±0.01	0.05±0.01	0.03±0.01
DHA/EPA	1.4±0.1 ^{a}	1.6±0.2 ^{b}	1.8±0.1 ^{b}	3.0±0.3 ^{c}	3.2±0.2 ^{c}

Means within rows bearing different letters are significantly different ($p < 0.05$). Statistical analyses was performed only for fatty acids > 1%, while fatty acids with a percentage < 1% were excluded because their concentrations were close to the limit of detection.

Specifically, the higher was the BSF prepupae meal dietary inclusion level, the lower was the n3 (28.1 ± 0.9, 26.1 ± 1.2, 23.7 ± 0.9, 21.6 ± 1.1 and 17.6 ± 0.6 % for Hi0, Hi25, Hi50, Hi75 and Hi100, respectively) and the higher was the n6 (13.0 ± 0.2, 14.1 ± 0.2, 14.9 ± 0.3, 17.6 ± 0.2 and 18.2 ± 0.3 % for Hi0, Hi25, Hi50, Hi75 and Hi100, respectively) percentages detected. In terms of n9 percentage, only Hi25 and Hi50 groups (17.7 ± 0.4 and 17.9 ± 0.3 %, respectively) showed significantly higher values compared to Hi0 group (17.3 ± 0.2 %). Finally, considering the n6/n3 ratio

(Fig. 2d), no significant differences were detected among Hi0, Hi25 and Hi50 groups (0.5 ± 0.1, 0.5 ± 0.1 and 0.6 ± 0.1, respectively), while Hi75 and Hi100 (0.8 ± 0.1 and 1.0 ± 0.1, respectively) showed significantly higher values compared to the other experimental groups.

Table 3 shows the fatty acids composition of total lipids of zebrafish larvae. The fatty acids profile of larvae fed the Hi0 diet was characterized by high percentages of palmitic (16:0), oleic (18:1n9), eicosapentaenoic (20:5n3; EPA), and docosahexaenoic (22:6n3; DHA) acids, followed by linoleic (18:2n6), palmitoleic (16:1n7) and stearic (18:0) acids. The increasing inclusion levels of BSF prepupae meal respect to FM in the diets triggered some changes in fatty acids composition of the larvae. A substantial (up to ~30-folds) increase in the lauric acid (12:0) and a significant decrease in EPA percentage were detected in fish fed diets containing increasing inclusion levels of BSF prepupae meal. In terms of DHA, a similar but milder trend was observed respect to EPA. Consequently, the DHA/EPA ratio significantly increased with the increasing dietary BSF prepupae meal inclusion levels respect to FM (Table 3).

Histology

Histological analyses were performed in order to detect possible inflammatory events in the intestine and to evaluate lipid accumulation or steatosis in the liver. The intestine mucosa appeared unaltered and did not show any appreciable inflammatory influx in all experimental groups and fish fed BFS-based diets showed a normal intestinal morphology, comparable to Hi0 group (Fig. 3a-j).

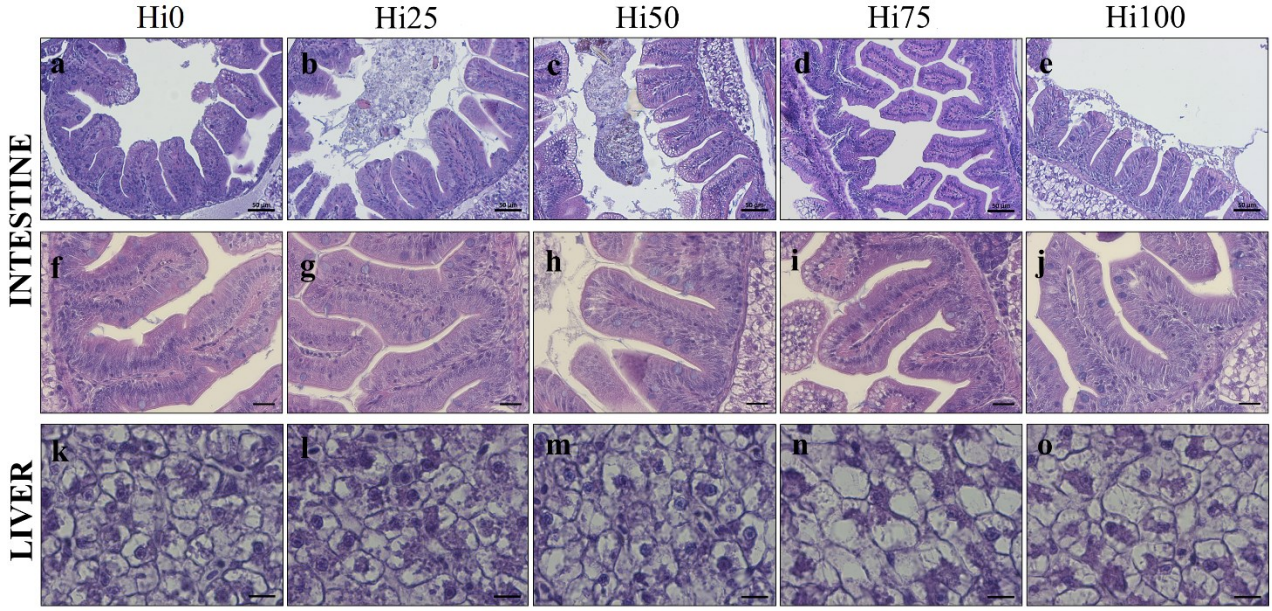

Figure 3. Example of histomorphology of intestine and liver from zebrafish larvae. (**a-j**) intestine; (**k-o**) liver. Scale bars: (**a-e**) 50 µm; (**f-j**) 20 µm; (**k-o**) 10 µm. Zebrafish larvae fed diets including 0, 25, 50, 75 and 100 % of BSF prepupae meal respect to FM (Hi0, Hi25, Hi50, Hi75 and Hi100, respectively).

Conversely, results evidenced a variable degree of lipid accumulation in the liver of the experimental groups (Fig. 3k-o). The most appreciable visual differences were observed in the Hi75 and Hi100 groups. Indeed, while all experimental groups presented a modest fat liver parenchyma, Hi75 and Hi100 showed a severe degree of steatosis with swollen hepatocytes and abundant intracytoplasmic lipid accumulation (Fig. 3n,o). These results were confirmed by the quantification of the percentage of fat fraction (PFF) on liver sections that showed a significant increase in response to dietary treatments (Fig. 4). In particular, Hi50 showed a significant increase in PPF (48.7 ± 2.4 %) compared to Hi0 and Hi25 (41.7 ± 2.1 and 42.5 ± 1.2 %, respectively) groups. However, the highest PFF values were detected in Hi75 and Hi100 (58.7 ± 0.9 and 60.6 ± 1.5 %, respectively) with a significant difference compared to the other groups.

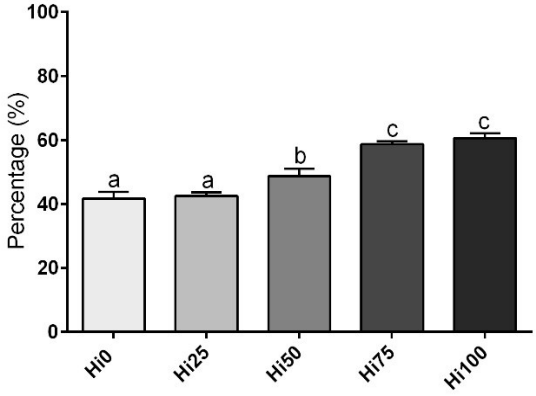

Figure 4. Percentage of fat fraction (PFF) in liver tissue calculated on histological sections.in Hi0, Hi25, Hi50, Hi75 and Hi100 groups. Values are presented as mean ± SD (n = 9). Different letters indicate statistically significant differences among the experimental groups. Zebrafish larvae fed diets including 0, 25, 50, 75 and 100 % of BSF prepupae meal respect to FM (Hi0, Hi25, Hi50, Hi75 and Hi100, respectively).

FTIR analysis

The average absorbance spectra of Hi0, Hi25, Hi50, Hi75 and Hi100 larval groups are shown in Figure 5. The analysis of IR spectra allowed to distinguish the vibrational modes of lipids, proteins, carbohydrates and phosphates (see Materials and Methods section) [**12**]. In terms of biochemical composition of zebrafish larvae samples belonging to the different dietary groups, the following considerations can be drawn: (i) significantly higher amounts of overall lipids (LIP/TBM, Fig. 6a) were detected in Hi50, Hi75 and Hi100 zebrafish larvae compared to Hi0 and Hi25 ones; (ii) in all zebrafish larvae fed on diets with different inclusion levels of BSF prepupae meal, a significant increase in SFA (1744/LIP and 2925/LIP, Fig. 6b and c) and a significant decrease in unsaturated ones (3010/LIP, Fig. 6d) were observed; (iii) in terms of protein composition, no significant changes were detected in the overall amount of proteins (PRT/TBM, Fig. 7a) among the experimental groups; (iv) a slight but significant decrease of collagen (1234/PRT, Fig. 7b) was observed in all zebrafish

larvae fed on diets including BFS prepupae meal, and (v) a higher amount of mucin (1055/PRT, Fig. 7c) was found in Hi50, Hi75 and Hi100 zebrafish larvae.

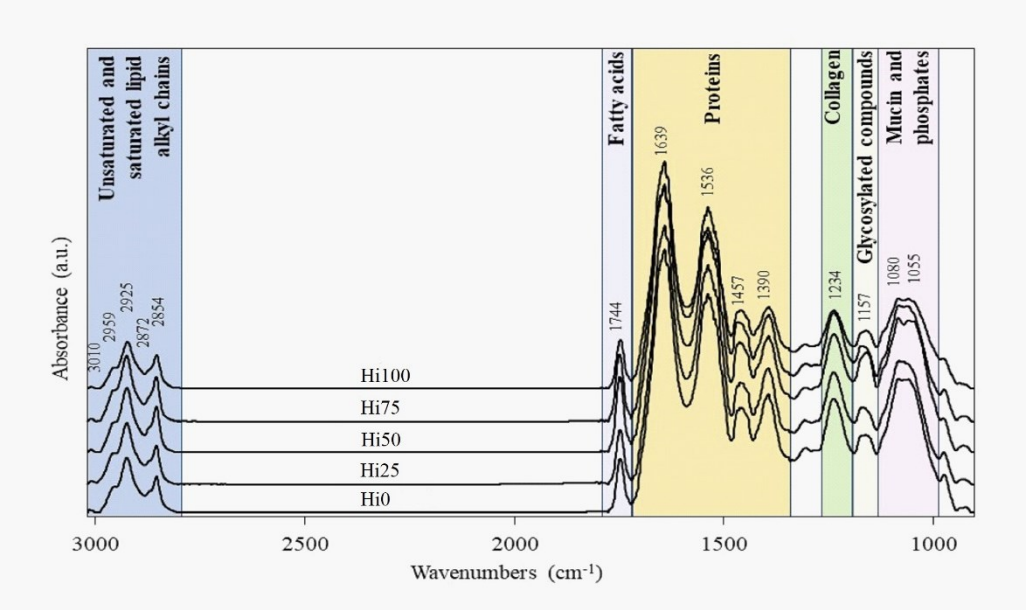

Figure 5. IR spectra of zebrafish larvae. Average absorbance spectra of zebrafish larvae fed diets including 0, 25, 50, 75 and 100 % of BSF meal (Hi0, Hi25, Hi50, Hi75 and Hi100). For clarity reasons, spectra are shifted along y-axis. The position (in terms of wavenumbers) of the most featuring IR peaks is reported, together with (coloured boxes) the corresponding biochemical meaning.

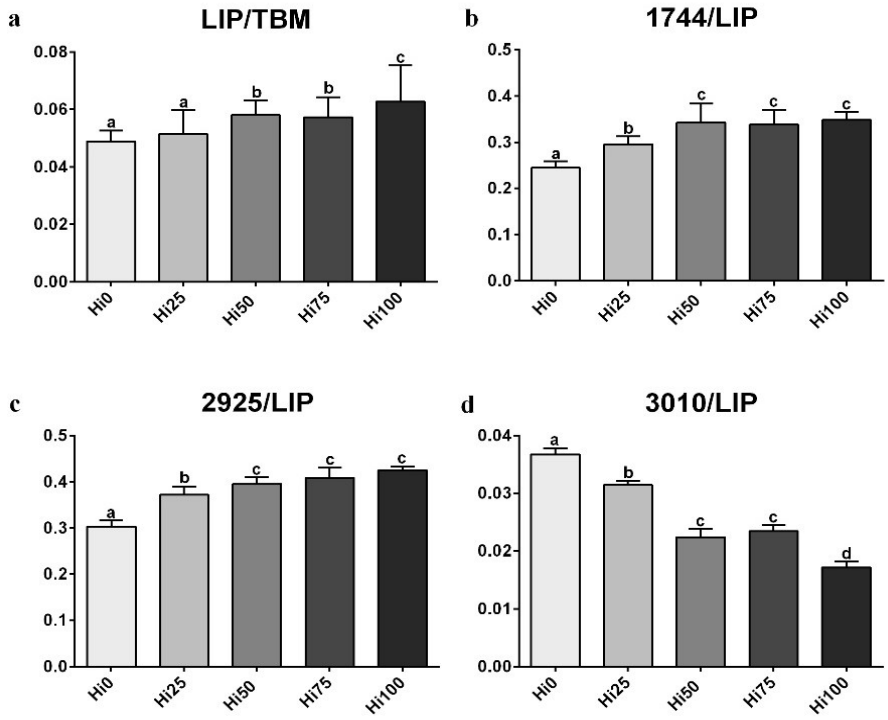

Figure 6. Statistical analysis of lipid composition. Numerical variation of IR band area ratios in zebrafish larvae fed diets including 0, 25, 50, 75 and 100 % of BSF prepupae meal (Hi0, Hi25, Hi50, Hi75 and Hi100): (**a**) LIP/TBM (overall amount of lipids); (**b**) 1744/LIP (amount of fatty acids with respect to lipids); (**c**) 2925/LIP (amount of SFA with respect to lipids) and (d) 3010/LIP (amount of unsaturated fatty acids with respect to lipids). Different letters indicate statistically significant differences among groups ($p < 0.05$).

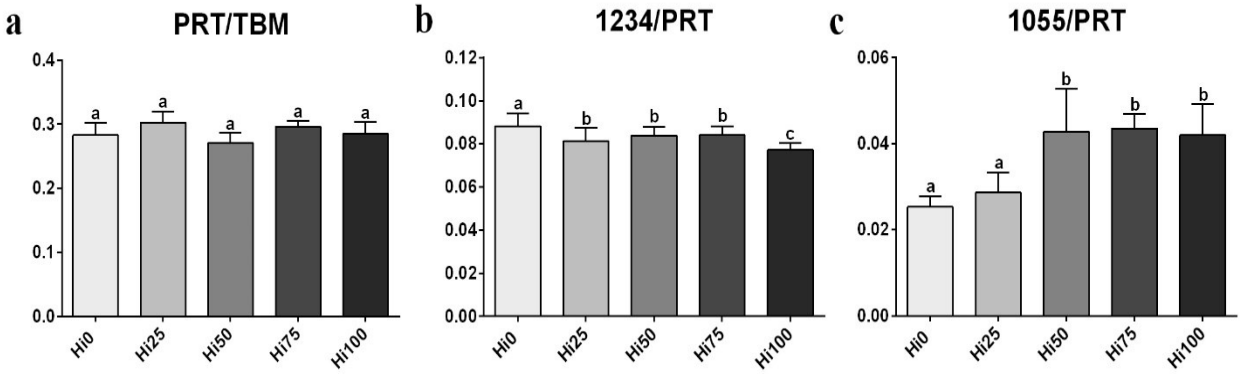

Figure 7. Statistical analysis of protein composition. Numerical variation of IR band area ratios in zebrafish larvae fed diets including 0, 25, 50, 75 and 100 % of BSF prepupae meal (Hi0, Hi25, Hi50, Hi75 and Hi100): (**a**) PRT/TBM (overall amount of proteins); (**b**) 1234/PRT (amount of collagen with respect to proteins) and (**c**) 1055/PRT (amount of mucin with respect to proteins). Different letters indicate statistically significant differences among groups ($p < 0.05$).

Microbiome

A total of 247,654 reads passed the filters applied through QIIME, with an average value of 49,530 reads/sample, and a mean sequence length of 464 bp. The Good's coverage indicated also satisfactory coverage for all samples (> 99%) (Table 4). Alpha-diversity indicated the highest number of OTUs after 50% of BSF prepupae meal inclusion if compared with Hi0 and with 25% of inclusion. Beta diversity calculation based on weighted and on unweight UniFrac distance matrix showed a clear separation of the Hi0 samples if compared with those from BSF-based diets (Fig. 8). Furthermore, 50 and 75% of BSF prepupae meal dietary inclusion respect to FM showed a similar effect on microbial composition. As shown in Figure 9, the main OTUs shared among the data sets were *Cetobacterium* that reached 50% of the relative abundance in sample GHi50 and about 30% in the other samples. *Vibrio* and Mycoplasmataceae decreased with the increasing dietary BSF prepupae meal inclusion levels respect to FM; *Flavobacterium* and *Plesiomonas* were present at very low abundance in GHi100 sample, whereas in the other samples the same two genera showed a remarkable presence. Finally, among other bacteria, Aeromonadaceae, *Ochrobactrum* and *Tetrathiobacter* were also detected.

Table 4. Observed diversity, good's coverage and number of sequences for the 16S rRNA amplicons obtained from zebrafish gut (G) samples

Sample	Good's coverage	PD_whole_tree	chao1	Observed_species	Shannon
GHi0	99.77	19.37	418.40	299	3.41
GHi25	99.88	13.26	294.02	259	4.01
GHi50	99.80	20.37	462.88	380	3.25
GHi75	99.75	21.23	470.88	388	3.49
GHi100	99.75	19.97	419.85	332	3.72

Zebrafish larvae fed diets including 0, 25, 50, 75 and 100 % of BSF prepupae meal respect to FM (Hi0, Hi25, Hi50, Hi75 and Hi100, respectively).

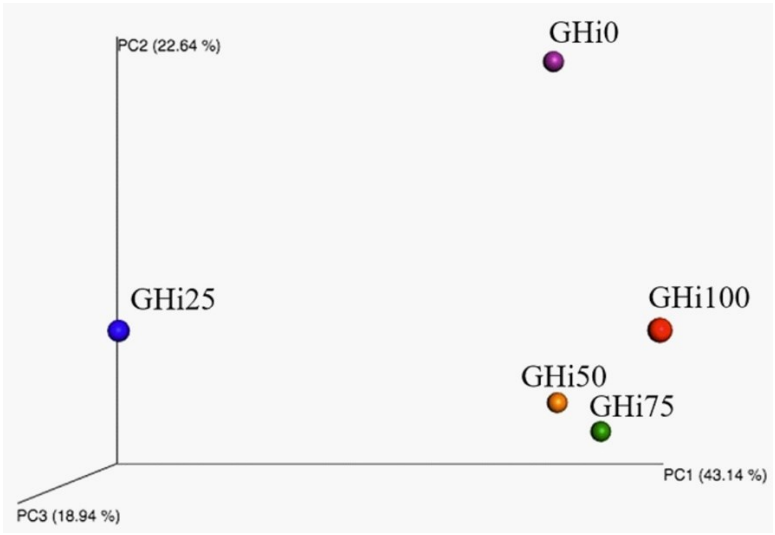

Figure 8. PCA based on the OTU abundance of the zebrafish samples grouped as a function of the amount of BSF prepupae meal included in the diet respect to FM. The first component (PC1) accounts for the 43.14% of the variance, the second component (PC2) accounts for the 22.64% of the variance, the third component (PC3) accounts for the 18.94% of the variance. Zebrafish larvae fed diets including 0, 25, 50, 75 and 100 % of BSF prepupae meal respect to FM (Hi0, Hi25, Hi50, Hi75 and Hi100, respectively).

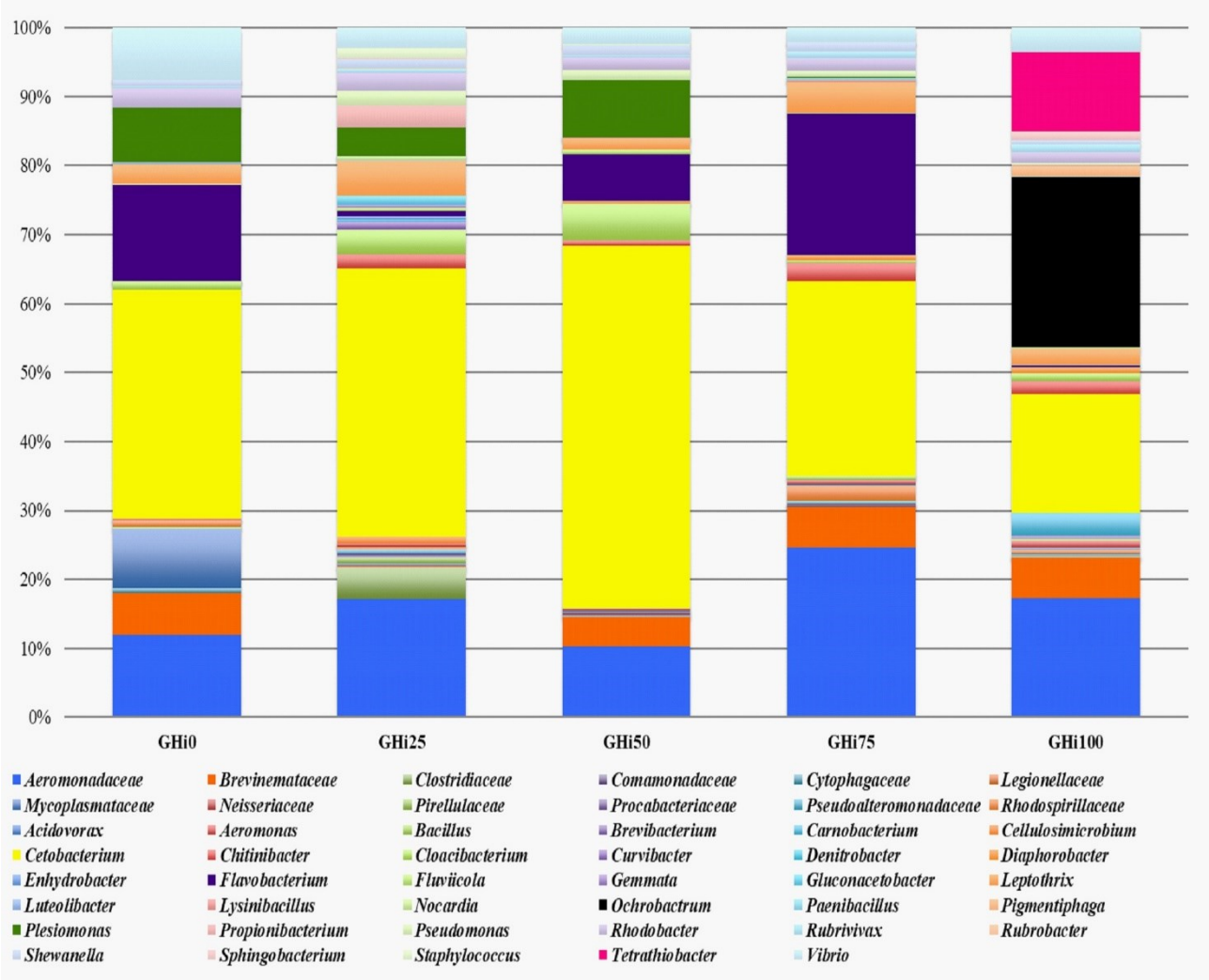

Figure 9. Relative abundances as identified by MiSeq Illumina expressed as the percent ratio between the sum of reads of each OTUs and the total number of reads found in the zebrafish gut (G) samples. Zebrafish larvae fed diets including 0, 25, 50, 75 and 100 % of BSF prepupae meal respect to FM (Hi0, Hi25, Hi50, Hi75 and Hi100, respectively).

Real-time PCR results

Growth factors. Higher mean levels in the expression of the *igf1* and *igf2a* genes (Fig. 10a,b) were detected in all experimental groups fed BSF-based diets compared to Hi0 group (with the exception of *igf1* gene expression in Hi25 group). In particular, Hi75 and Hi100 groups showed significantly higher values compared to Hi25 group in terms of *igf1* gene expression, and to Hi0 in terms of *igf2a* gene expression. Only Hi50 and Hi100 groups showed significantly higher levels of *mstnb* gene expression than Hi0 (Fig. 10c).

Stress response. Considering stress markers (*nr3c1* and *hsp70.1*; Fig. 10d,e), all groups fed BSF-based diets showed a significantly higher gene expression compared to Hi0 group (with the exception of *nr3c1* gene expression in Hi25 group). Furthermore, no significant differences were evident among Hi50, Hi75 and Hi100 groups, while Hi25 group showed significantly lower values compared to groups fed diets with higher BSF prepupae meal inclusion levels respect to FM (with the exception of *nr3c1* gene expression in Hi50 group).

Lipid metabolism. The highest BSF prepupae meal dietary inclusion (Hi100) caused the highest expression of the genes involved in long-chain PUFA elongation (*elovl2* and *elovl5*) and desaturation (*fads2*). As illustrated in Figure 10f-h, Hi100 group presented the highest gene expression compared to the other experimental groups. More specifically, no significant differences in the *elovl2* gene expression of the were detected between Hi0 and Hi25 group (Fig. 10f), while both Hi50 and Hi75 showed a significantly higher expression than Hi0. No significant differences in the *elovl5* and *fads2* gene expression of the (Fig. 10g,h) were observed among Hi0, Hi25, Hi50 and Hi75 groups.

Appetite. As shown in Figure 10i,j, the higher was the BSF prepupae meal dietary inclusion, the higher was the *ghrl* and *npy* gene expression. However, no significant differences were observed between Hi0 and Hi25 in terms of *ghrl* gene expression and among Hi0, Hi25 and Hi50 in terms of *npy* gene expression. Figure 10k shows *cnr1* gene expression. No BSF prepupae meal dose dependency was observed in the expression of this specific gene, since only Hi25 and Hi50 groups evidenced significantly higher values than Hi0. Groups fed the highest BSF prepupae meal dietary inclusions respect to FM (Hi75 and Hi100) showed significantly higher *lepa* gene expression (Fig. 10l) compared to the other groups, while no significant differences were detected among Hi0, Hi25 and Hi50 groups.

Immune response. Considering genes involved in the immune response, higher BSF prepupae meal dietary inclusions respect to FM resulted in a significantly higher gene expression compared to Hi0.

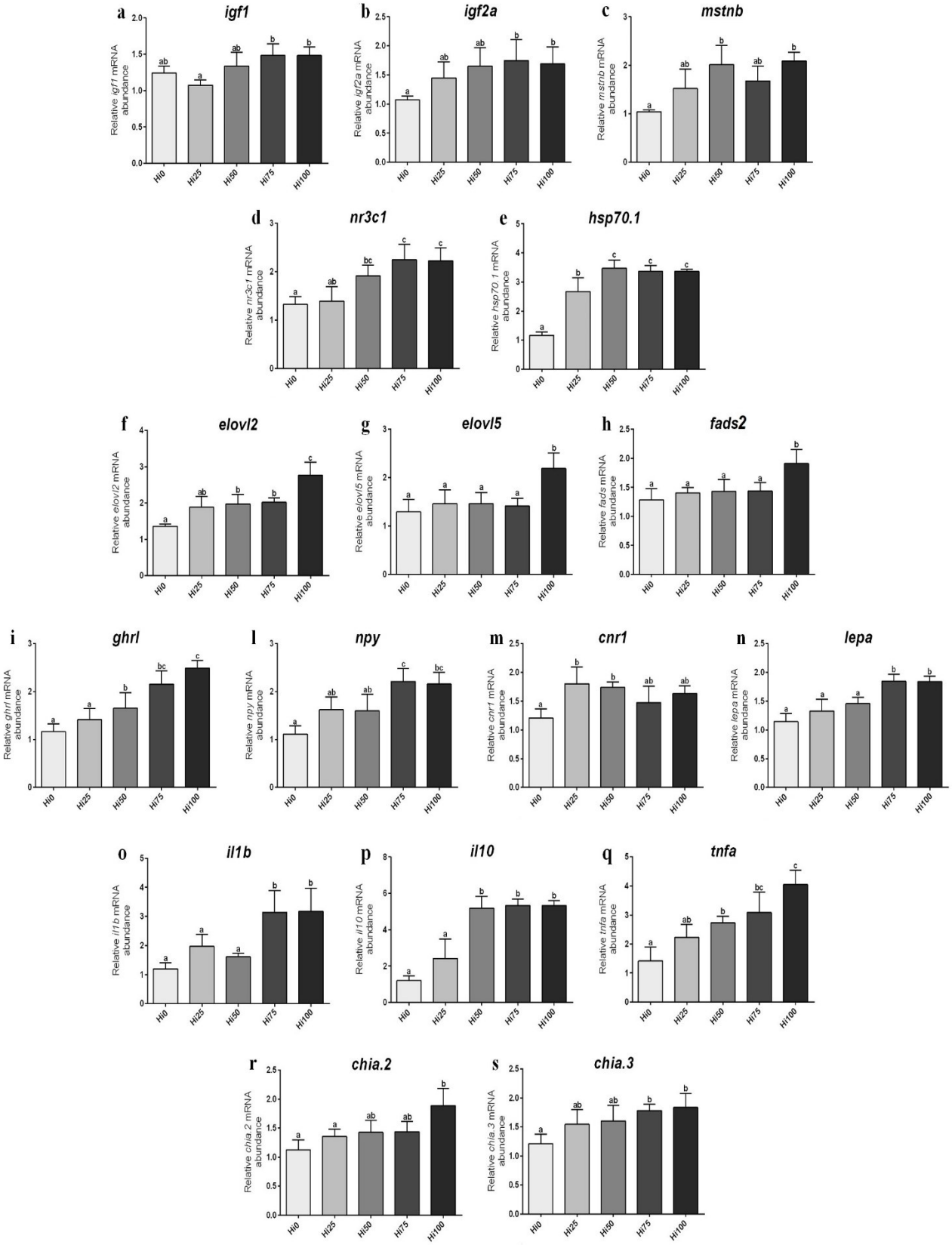

Figure 10. Relative mRNA levels of genes analysed in zebrafish larvae. (**a**) *igf1*, (**b**) *igf2a*, (**c**) *mstnb*, (**d**) *nr3c1*, (**e**) *hsp70.1*, (**f**) *elovl2*, (**g**) *elovl5*, (**h**) *fads2*, (**i**) *ghrl*, (**j**) *npy*, (**k**) *cnr1*, (**l**) *lepa*, (**m**) *il1b*, (**n**) *il10*, (**o**) *tnfa*, (**p**) *chia.2*, (**q**) *chia.3*. Different letters indicate statistically significant differences among experimental groups compared within the same sampling time ($p < 0.05$). Values are presented as mean ± SD (n = 5). Zebrafish larvae fed diets including 0, 25, 50, 75 and 100 % of BSF prepupae meal respect to FM (Hi0, Hi25, Hi50, Hi75 and Hi100, respectively).

Specifically, no significant differences were evident in the *il1b* gene expression (Fig. 10m) among Hi0, Hi25 and Hi50 groups, while Hi75 and Hi100 groups showed significantly higher values compared to the other experimental groups. Similarly, Hi50, Hi75 and Hi100 groups showed a significantly higher *il10* gene expression (Fig. 10n) compared to Hi0 and Hi25 groups, which did not differ significantly from each other. Finally, as reported in Fig. 10o, the increasing BSF prepupae meal dietary inclusion levels respect to FM resulted in a significant dose-dependent increase in *tnfa* gene expression.

Chitinases. All the experimental groups fed BSF-based diets showed an increase in chitinases gene expression (Fig. 10p,q). Specifically, no significant differences were evident in *chia.2* and *chia.3* gene expression among all the experimental groups, with the exception of *chia.2* in Hi100 group and *chia.3* in both Hi75 and Hi100 groups, which were significantly higher than Hi0 group.

Discussion

The use of insects in aquafeed still faces possible limitations because of their lack in PUFA [**49**] and presence of chitin [**86**,**87**]. Insects are usually farmed on vegetable organic by-products, and previous studies showed that the final insect biomass had a very low PUFA and a high SFA content [**12**,**36**]. However, it is now well established that the quantity and quality of fatty acids in insects can be modified by the growth substrate [**22**,**65**,**87**]. Specifically, in the present study we demonstrated that the addition of a 10% (w/w) of *Schizochytrium* sp. to the growth substrate is an efficient approach to increase the PUFA content of the final insect biomass. This "enrichment" procedure of the insect biomass caused a progressive increase in PUFA content in the Hi25, Hi50, Hi75 and Hi100 diets, respectively, highlighting that the enrichment method used is appropriate and represents, in this sense, an interesting and innovative approach on how food by-products produced on land (coffee silverskin) can be enriched and bio-converted into a valuable biomass for aquafeed production [**89**,**90**].

Compared to previous insect-based diets already tested on zebrafish [**12**,**91**], the ones tested in this study showed good performances on fish growth, since higher BSF prepupae meal inclusion levels in the experimental diets resulted in higher larval SGR%. This result was supported by the analysed molecular markers of growth but is in contrast with previous studies reporting that BSF meal inclusion levels higher than 40% often resulted in negative effects on fish growth and welfare [**66**,**92**,**93**]. In particular, previous studies evidenced that such high inclusion levels affected both intestine and liver integrity and, thus, dietary nutrients absorption [**23**,**37**,**94**].

The different diets tested in the present study also affected the fatty acids composition of zebrafish larvae. However, differences among fatty acids classes were less evident in zebrafish larvae compared to those detected in the diets. In fact, as a freshwater species, zebrafish are able to convert shorter-

chain fatty acids in highly unsaturated ones through the elongation and desaturation pathways [**42**]. This was clearly documented in the present study by a higher *elovl2*, *elovl5* and *fads2* gene expression in all the experimental groups fed on BSF-based diets (with emphasis on the Hi100 group).

Gas chromatographic results were also confirmed and integrated by FTIR analyses which provided data about the macromolecular composition of the analysed biological samples [**95**]. Specifically, FTIR analyses showed that there were no differences in the total amount of proteins (PRT/TBM ratio) (with the exception of collagen, represented by 1234/PRT ratio, that slightly decreased) among the experimental groups, highlighting that BSF prepupae meal is a valuable protein source for aquafeeds [**34**,**96**,**97**]. Concerning lipids, the same analyses revealed that the increase of BSF prepupae meal percentage in the diets was associated with a general increase in total larval lipid content. Additionally, analysing specific wavelengths, FTIR confirmed the gas chromatographic results, evidencing an overall increase in SFA (1744/LIP and 2925/LIP ratios) and a decrease in unsaturated fatty acids (3010/LIP ratio).

Molecular markers related to the appetite stimulus were fully supported by the biometric results. Fish food intake is regulated by specific regions in the brain that interpret and integrate positive (orexigenic) and negative (anorexigenic) signals derived from the hypothalamic area (neurohormones) and from the body periphery [**98**,**99**]. Specifically, the orexigenic signals *ghrl* and *npy* analysed in the present study, showed a dose-dependent gene expression increase related to the BSF prepupae meal inclusion levels in the diets (the higher the BSF prepupae meal inclusion level, the higher their gene expression), while the *cnr1* gene expression was always higher in all the larval groups fed on BSF-based diets. Conversely, the results obtained from the anorexigenic signal *lepa* was not obvious. Leptin is usually involved in the inhibition of orexigenic pathways and in the stimulation of anorexigenic [**100**,**101**]; however, in the present study, an opposite activity was evidenced. This anomalous pattern could be explained by the FTIR analyses that revealed an increasing total lipid content (LIP/TBM ratio) in larvae fed on diets with increasing BSF prepupae meal levels. In fact, previous studies, performed both on mammals and fish species, demonstrated a positive correlation between leptin levels and amount of adipose tissue [**102**,**103**,**104**]. Alternatively, an interconnection between leptin and proinflammatory cytokines exist [**105**]. Leptin is known to enhance the production of proinflammatory cytokines, as well as proinflammatory cytokines are able to trigger leptin release [**106**]. In the present study, the increasing *lepa* gene expression observed in larvae fed on BSF-based diets might have promoted the immune-response observed in the same experimental groups. However, it should be mentioned that all BSF-based diets contain chitin [**107**,**108**], a molecule which still has a controversial role in aquafeeds. Indeed, some studies reported a beneficial modulatory role of chitin on fish immune system and microbiota [**109-113**], while others

showed that, especially at high inclusion levels, chitin may induce a reduction in nutrient assimilation in the intestinal tract [**86**,**114**,**115**]. In support of this conclusion, Hi50, Hi75 and Hi100 larvae showed a significant increase of mucin (1055/PRT ratio), possibly associated to a higher intestine lubrication necessary for a proper intestinal transit of these diets.

In the present study, no specific inflammatory events were detected through the histological analysis of the intestine samples, suggesting: (i) a possible digestion of chitin by zebrafish larvae through specific chitinases (*chia.2* and *chia.3*); (ii) a possible intestinal anti-inflammatory role of lauric acid (12:0). This fatty acid, which in the present study increased its dietary amount with the increasing BSF prepupae meal inclusion in the diets, has been demonstrated to have beneficial effects on fish gut welfare by mitigating inflammatory conditions [**91**].

The histological analysis of the liver showed a lipid accumulation in the hepatic parenchyma in all analysed samples, regardless of the dietary treatment. Specifically, the Hi75 and Hi100 groups showed a severe level of hepatic steatosis, a pathological condition that has previously been related to a high n6/n3 ratio [**36**,**91**]. This hepatic disorder is probably the cause of the higher gene expression of the stress markers (*nr3c1* and *hsp70.1*).

Finally, insects are known to possess natural bioactive molecules that are known to modulate fish microbiota [**55**,**56**] and, therefore, the bacterial community is expected to impact host metabolism and health status. Although autochthonous bacterial communities were dominated by the same phyla regardless of the diet, the present study showed that bacterial populations are dynamic and can be modulated by dietary inclusion of insect meal. Some bacteria were almost present in all the samples analysed, while others were mainly modulated by the BSF-based diets. Among the ubiquitous species, the massive presence of *Cetobacterium* in all gut samples suggested that graded BSF prepupae meal inclusion levels in the diets did not influence the presence of such a fish associated microorganism. In fact, *Cetobacterium* has already been found as core genus in the gut of zebrafish with beneficial effect on fish health because of its ability to produce cobalamin (vitamin B12) [**116-119**]. Mycoplasmataceae were also dominant in the present study and have already been found among the dominant microorganisms in fish intestines, with a possible beneficial action on host health by producing lactic acid with antibacterial effect [**50**]. Aeromonadaceae were also detected in all analysed gut samples, irrespective of the diet used. This bacterial family has already been found as naturally associated with the gut of fish or insects [**50**,**120**,**121**], suggesting a contribution of BSF-based diets in the occurrence of such a bacterial family in the gut of the analysed zebrafish. BSF meal inclusion in the diets modified the presence of some bacteria. Specifically, the presence of *Vibrio* was negatively influenced by the inclusion of increasing graded levels of BSF prepupae meal respect to FM in the diets. As reported by Brugman et al. [**122**], in the zebrafish intestine, T lymphocytes can

control the outgrowth of *Vibrio* species. As shown by the Real Time PCR analyses, the supply of BSF-based diets stimulated immune-response related genes that might be implied in *Vibrio* species control. However, further research is needed to better understand the involvement of BSF prepupae meal in this aspect. Finally, both *Ochrobactrum* and *Tetrathiobacter* were detected in the samples GHi100. The occurrence of *Ochrobactrum* was already reported in zebrafish gut [**123**] as well as in the microbiota of insects as coleoptera and lepidoptera [**120**], while *Tetrathiobacter* has already been isolated from the midgut of ticks [**124**]. These data suggest that the inclusion of BSF prepupae meal in fish diets can contribute to modify the fish microbiota with a specific possible involvement in the allochthonous microbiota modification. As a general remark about gut microbiota, bacterial diversity is considered a positive indicator of gut health while a reduced diversity is often associated to dysbiosis and risk of disease in fish [**55**]. In the present study, the PCA analyses revealed a difference between the Hi0-Hi25 and Hi50-Hi75-Hi100 groups, thus suggesting a possible influence of BSF-based diets on the zebrafish gut microbiota, to be further investigated.

As a conclusion, BSF biomass should be enriched in PUFA in order to meet aquafeed requirements. The present study showed that the addition of a 10% *Schizochytrium* sp. to the growth substrate is a valid method to achieve this objective. Furthermore, the present study evidenced that a 50% BSF meal inclusion level in the diet sustains a better fish growth and does not have any major negative effects on the fish. Higher inclusion levels affect larval liver histology and induce a general increase in lipid accumulation and stress response. This novel approach represents an interesting example of how, in the long term, a circular economy applied to the aquaculture sector may sustain animal's welfare and encourage sustainability and competitiveness.

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3.2

Zebrafish (*Danio rerio*) physiological and behavioural responses to insect-based diets: a multidisciplinary approach

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Abstract

Black Soldier Fly (BSF) prepupae meal is considered as an alternative, emerging and sustainable ingredient for aquafeed production. However, results on fish physiological responses are still fragmentary and often controversial, while no studies are available on fish behaviour in response to these new diets. The present work represents the first comprehensive multidisciplinary study aimed to investigate zebrafish physiological and behavioural responses to BSF-based diets. Five experimental diets characterized by increasing inclusion levels (0, 25, 50, 75 and 100 % respect to fish meal) of full fat BSF prepupae meal were tested during a 2-month feeding trial. Prepupae were cultured on coffee silverskin growth substrate enriched with a 10% *Schizochytrium* sp. to improve insects' fatty acids profile. The responses of zebrafish were assayed through biometric, histological, gas chromatographic, microbiological, spectroscopic, molecular and behavioural analyses. Results evidenced that BSF-based diets affected fish fatty acids composition, while behavioural tests did not show differences among groups. Specifically, a 50% BSF inclusion level diet represented the best compromise between ingredient sustainability and proper fish growth and welfare. Fish fed with higher BSF inclusions (75 and 100 %) showed hepatic steatosis, microbiota modification, higher lipid content, fatty acids modification and higher expression of immune response markers.

Introduction

The promotion of high-quality fish production as well as fish welfare are the main aquaculture goals, both strictly related to an adequate fish nutrition [**1**]. The use of fish meal (FM) and fish oil (FO) in aquafeed formulation is no more feasible because of important environmental and economic issues [**2**]. Over the last decades, several alternative ingredients (plant origin proteins, microalgae and processed animal proteins) to FM and FO have been tested [**3**]. However, each of these ingredients showed some disadvantages in its application in aquafeed formulation including unbalanced amino acid profile, poor protein digestibility, presence of anti-nutritional factors and high production costs [**3**,**4**].

Insects are now considered as an alternative and sustainable ingredient for feed production [**5**]. In particular, the Black Soldier Fly (*Hermetia illucens*; BSF) larvae are one of the most promising candidates because of their proper protein content and the amino acid composition similar to that of FM [**6**,**7**]. In addition, BSF have low environmental requirements, a high feed conversion efficiency and they can growth on organic by-products, promoting sustainability and the circular economy concept in the aquaculture sector [**8**,**9**]. Several studies tested different BSF inclusion levels in aquafeed formulation but results on fish physiological responses are still controversial, while behavioural effects on fish are completely missing [**10-13**]. The use of BSF in aquafeed has been

shown, at certain inclusion levels, to improve gut health, immunity and general fish welfare [**14**]. As an example, BSF prepupae meal contains lauric acid and chitin, which showed, at certain concentrations, anti-inflammatory and immune-boosting properties [**14**,**15**]. Additionally, BSF-based diets have been proven to increase biodiversity in fish microbiome community structure [**16-18**], which in turn, has an important role in host metabolism, nutrition, immunity and welfare [**18**,**19**].

However, the use of insect meal in aquafeed formulation still faces some bottlenecks [**5**]. Most insect species, including BSF, are rich in saturated fatty acids (SFA) and contain negligible amounts of polyunsaturated (PUFA) ones [**20**]. A high dietary SFA content, coupled with a high n6/n3 ratio, has been shown to play a key role in fish hepatic steatosis development [**13**,**21**]. Furthermore, insect meal may alter the biochemical characteristics of fish fillet, especially regarding the fatty acids composition, even in absence of major alterations of its sensory properties [**12**,**22**]. While a diet rich in n3 fatty acids has generally been associated with a lower risk to develop neurodegenerative and cardiovascular diseases in humans and animal models [**23**,**24**], the chronic ingestion of n6 and SFA has been related to wide loss of brain volume and synapses [**25**] and to behavioural impairments such as dementia [**26**]. Impaired behaviour and learning have also been related to a reduction in gut microbiota variability [**27**], a condition often related to a n6 and SFA rich diet [**28**].

Given their unbalanced fatty acids profile, BSF-based diets could have direct effect on fish welfare and neuronal functioning. At this regard, the fatty acids composition of BSF larvae can be modified by the growth substrate [**6**]. Specifically, Truzzi et al. [**9**] and Zarantoniello et al. [**19**] demonstrated that culturing BSF larvae on an organic substrate enriched with 10% *Schizochytrium* sp. significantly increased insects' PUFA content. With respect to previous studies carried out on zebrafish [**21**,**29**], this enriching procedure allows to include up to 50% of BSF meal in the fish diet without impairing fish physiology, and hence represents a remarkable example of sustainable circular economy. Zebrafish represents an ideal organism to better understand fish physiological responses to new ingredients [**30**] and an emerging useful model organism for neuroscience/behavioural research [**31**].

The present work represents the first comprehensive multidisciplinary study aimed to investigate zebrafish physiological and behavioural responses to BSF-based diets. Five experimental diets characterized by increasing inclusion levels of full-fat BSF prepupae meal with respect to FM were tested during a two-months feeding trial. BSF prepupae were cultured on a coffee silverskin growth substrate enriched with a 10% *Schizochytrium* sp. to improve insects' fatty acids profile [**9**]. The physiological responses of zebrafish were analysed through biometric, histological, gas chromatographic, microbiological, spectroscopic and molecular analyses. Behaviour was assessed with an open-field exploration test and a standard photic entrainment test.

Methods

Ethics

All procedures involving animals were conducted in line with the Italian legislation and approved by the Ethics Committee of Università Politecnica delle Marche and the Italian Ministry of Health (626/2018-PR)

Insects rearing and fish diet production

Insects rearing and fish diet production were performed according to Zarantoniello et al. [**20**] (chapter 3.1). Specifically, the insect diet was formulated including a 10% (w/w) of *Schizochytrium* sp. to the coffee by-product. The freeze-dried *Schizochytrium* sp. was provided by AlghItaly Società Agricola S.R.L. (Sommacampagna, Verona, Italy). Distilled water was added to feeding substrate to reach a moisture of ~70%. The insect rearing was carried out in a climatic chamber at a 27 ± 1 °C temperature, relative humidity of 65 ± 5 %, in continuous darkness. Six days old larvae (purchased from Smart Bugs s.s. Ponzano Veneto, Treviso, Italy) were hand counted and divided in groups of 640 larvae per replicate (n = 65) for a total of 41,600 specimens. Each replicate consisted of a plastic box (57 × 38 ×16 cm) screened with fine-mesh cotton gauze and covered with a lid provided with 90 ventilation holes of 0.05 cm of diameter. Each larva was provided with a feeding rate of 100 mg/day. Food was prepared and added once a week (448 g for each box). Insects were visually inspected every day and when prepupae were identified by the change in tegument colour from white to black, they were collected, washed, dried and stored at -80 °C.

Full-fat BSF prepupae were freeze dried, grinded with Retsch Centrifugal Grinding Mill ZM 1000 (Retsch GmbH, Haan, Germany) and used to prepare the experimental diets. A control diet (Hi0) containing FM, wheat gluten, pea protein concentrates and FO as major ingredients, was prepared according to a commercially available standard diet for zebrafish (Zebrafeed, Sparos ltd, Olhão, Portugal). The experimental diets were iso-nitrogenous (50%) and iso-lipidic (13%). BSF-based diets were prepared by including graded levels of BSF prepupae meal respect to FM (25, 50, 75, and 100 %, referred as Hi25 and Hi50, Hi75 and Hi100, respectively) in the Hi0 formulation. The obtained diets were then grinded and stored in under vacuum bags at -20 °C until used. Feed samples were analysed for moisture (AOAC #950.46), crude protein (AOAC #976.05), ash (AOAC #920.153) and ether extract (AOAC #991.36) contents according to AOAC [**32**]. The total lipid fraction of the test diets was extracted using chloroform-methanol (2:1 v:v) (Merck KGaA, Darmstadt, Germany) mixture according to Folch's method [**33**]. Diet formulation and proximate composition are shown in Table 1.

Table 1. Ingredients (g/kg) and proximate composition (g/100 g) of the experimental diets used in the present study.

	Hi0	Hi25	Hi50	Hi75	Hi100
Ingredients (g/kg)					
Fish meal [1]	470	400	250	110	-
Vegetable mix [2]	220	230	298	385	440
BSF prepupae meal	-	115	235	350	460
Wheat flour [3]	198	172	120	110	72
Fish oil	80	51	25	10	-
Soy lecithin	8	8	8	11	4
Mineral and Vitamin supplements [4]	14	14	14	14	14
Binder	10	10	10	10	10
Proximate composition (%)					
Dry Matter	97.1 ± 0.1	95.8 ± 0.1	94.9 ± 0.1	93.6 ± 0.1	92.7 ± 0.1
Crude protein	51.6 ± 0.1	50.7 ± 2.6	50.4 ± 0.3	51.2 ± 1.5	50.5 ± 3.1
Ether extract	14.4 ± 0.6	13.1 ± 0.4	12.9 ± 0.4	13.2 ± 0.5	13.0 ± 0.5
Nitrogen-free extract	21.3 ± 0.3	20.8 ± 1.0	20.6 ± 0.5	19.0 ± 0.7	18.5 ± 1.3
Ash	9.8 ± 0.2	11.1± 0.01	11.0 ± 0.0	10.1 ± 0.1	10.7 ± 0.1

[1] Raw ingredient kindly supplied by Skretting Italia. **[2]** Vegetable mix (pea protein concentrate: wheat gluten, 0.7:1 w/w) Lombarda trading srl, Casalbuttano & Uniti (Cremona, Italy) and Sacchetto spa (Torino, Italy). **[3]** Consorzio Agrario (Pordenone, Italy). **[4]** Mineral and Vitamin supplement composition (% mix): $CaHPO_4.2H_2O$, 78.9; MgO, 2.725; KCl, 0.005; NaCl, 17.65; $FeCO_3$, 0.335; $ZnSO_4.H_2O$, 0.197; $MnSO_4.H_2O$, 0.094; $CuSO_4.5H_2O$, 0.027; Na_2SeO_3, 0.067; thiamine hydrochloride (vitamin B1), 0.16; riboflavin (vitamin B2), 0.39; pyridoxine hydrocloride (vitamin B6), 0.21; cyanocobalamine (vitamin B12), 0.21; niacin (vitamin PP or B3), 2.12; calcium pantotenate, 0.63; folic acid, 0.10; biotin (vitamin H), 1.05; myo-inositol (vitamin B7), 3.15; stay C Roche (vitamin C), 4.51; tocopherol (vitamin E), 3.15; menadione (vitamin K3), 0.24; retinol (vitamin A 2500 UI/ kg diet), 0.026; cholecalciferol (vitamin D3 2400 UI/kg diet), 0.05; choline chloride, 83.99. For proximate composition, values are reported as mean of triplicate analyses.

Fish

Zebrafish AB embryos were maintained for 48 h in a Tecniplast system (Varese, Italy) in the following conditions: 28 °C temperature, pH 7.0, NH_3 and NO_2^- concentrations < 0.01 mg/L, NO_3^- concentration < 10 mg/L, and photoperiod 12L/12D, respectively. After this period, embryos were gently collected, counted under a stereomicroscope and randomly divided in five experimental groups (in triplicate) according to the five test diets.

Experimental design

Zebrafish larvae were initially reared in fifteen 20L tanks to set up the five experimental dietary treatments; each experimental group was composed of 1500 larvae (n = 3). The water in the larval tanks had the same chemical-physical characteristics of the parent's tank and was gently replaced 10 times a day by a dripping system [**34**]. After 30 days post fertilization (dpf), fish of each tank were gently transferred in bigger tanks (80L; 15 in total, 3 per each dietary group) equipped with mechanical and biological filtration (Panaque, Capranica, Viterbo, Italy). All the tanks were siphoned 30 min after feeding to remove possible feed excess and dead specimens. The required fish were

sampled at 60 dpf, euthanized with a lethal dose of MS222 (1 g/L; Merck KGaA) and properly stored for further analyses.

Feeding schedule

The duration of the feeding trial was 57 days. Starting from 5 to 60 dpf, zebrafish were fed as follows: (i) Hi0 group: larvae fed the diet including 0% of full-fat BSF prepupae meal (Hi0 diet); (ii) Hi25, Hi50, Hi75, Hi100 groups: larvae fed the diet including 25, 50, 75, 100 % respectively of full-fat BSF prepupae meal respect to FM. Feed particle sizes were < 100 μm from 5 to 15 dpf, 101-200 μm from 16 to 30 dpf and 201-400 μm from 31 to 60 dpf. Zebrafish were fed the experimental diets (2% body weight) twice a day and, in addition, from 5 to 10 dpf, all groups were fed (one feeding in the morning) on the rotifer *Brachionus plicatilis* (5 ind/mL) according to Lawrence et al. [**35**].

Biometry

For growth measurements, 60 fish per dietary group (20 fish per tank in triplicate) were randomly collected from the different tanks at hatching (3dpf) and at the end of experiment (60 dpf). At 3 dpf, wet weight was measured on pools of five larvae, while at 60 dpf fish were individually measured. The wet weight was determined by an analytical balance (precision: 0.1 mg). Specific growth rate (SGR%) was calculated as follows: $SGR\% = [(\ln W_f - \ln W_i) / t] \times 100$, where W_f is the final wet weight, W_i, the initial wet weight, and t, the number of days (57). Survival was evaluated at the end of the experiment (60 dpf) by counting the number of fish respect to the initial larvae.

Fatty acids composition

The experimental diets and fish deprived of the viscera (5 fish per tank, 15 per dietary group) were analysed for fatty acids composition. Samples were minced and homogenized (homogenizer MZ 4,110, DCG Eltronic, Monza, Italy), and freeze-dried (Edwards EF4, Crawley, Sussex, England). Lipid extraction was carried out with the Folch's method [**33**] for experimental diets and with Microwave-Assisted Extraction (MAE) for fish [**36**]. All lipid extracts were evaporated under laminar flow inert gas (N_2) until constant weight and re-suspended in 0.5 ml of n-epthane. Fatty acids methyl esters (FAME) were prepared according to Canonico et al. [**37**] using methyl ester of nonadecanoic acid (19:0; Dr. Ehrenstorfer GmbH, Augsburg, Germany) as internal standard. FAME were determined by an Agilent-6890 GC System (Milano, Italy) coupled to an Agilent-5973 N quadrupole Mass Selective Detector (MS) (Milano, Italy). A CPS ANALITICA CC-wax-MS (30 m × 0.25 mm ID, 0.25 μm film thickness) capillary column was used to separate FAME. Instrumental conditions

for the studied matrices were set up, according to Truzzi et al. [**38**]. For each analysed sample, at least three runs were performed on the GCMS.

Histology

Intestines and livers collected from 15 different fish specimens from dietary group (5 fish per tank in triplicate) were randomly collected and processed according to Cutrignelli et al. [**39**]. Samples were fixed by immersion in Bouin's solution (Merck KGaA) and then stored at 4°C for 24 h. Samples were washed three times with ethanol (70%) for ten minutes and preserved in the same ethanol solution. Samples were then dehydrated in graded ethanol solutions (80, 95 and 100 %), washed with xylene (Bio-Optica, Milano, Italy) and embedded in paraffin (Bio-Optica). Solidified paraffin blocks were cut with a microtome (Leica RM2125 RTS, Nussloch, Germany) and 5 µm sections were stained with Mayer hematoxylin and eosin Y (Merck KGaA). Sections were observed using a Zeiss Axio Imager.A2 (Zeiss, Oberkochen, Germany) microscope in order to study the hepatic parenchyma and intestine morphology. Images were acquired by mean of a combined color digital camera Axiocam 503 (Zeiss). Moreover, to ascertain the degree of fat accumulation in liver, a quantitative analysis was performed on a significant number of histological sections per liver samples (5 samples per tank, 15 per dietary group). Not evaluable areas such as blood vessels were not considered. The percentage of fat fraction (PFF) was calculated by mean of the ImageJ software setting a homogeneous threshold value.

FTIR measurements

Brains and livers from 15 fish specimens for dietary group (5 fish per tank in triplicate) were collected, quickly dissected and immediately frozen at -80 °C. Samples were then prepared for infrared spectroscopy (IR) measurements as follows.

Brain samples. FTIR measurements of brain samples were carried out by using a Perkin Elmer Spectrum GX1 spectrometer (Waltham, Massachusetts, USA). Brain samples of each dietary group were pooled, minced, homogenized (homogenizer MZ 4110, DCG Eltronic) and freeze-dried (Edwards EF4). Then, homogenized samples were deposited onto the crystal of the U-ATR accessory for the analysis in reflectance mode. For each sample, 10 spectra were collected in the 4000-650 cm^{-1} spectral range with a spectral resolution of 4 cm^{-1}; each spectrum was the result of 64 scans. Before each sample acquisition, a background spectrum was collected on the empty crystal. Raw IR spectra were converted in absorbance and corrected for the contribution of atmospheric carbon dioxide and water vapor (OPUS 7.1 software package, Bruker Optics GmbH, Ettlingen, Germany).

On these pre-processed spectra, the integrated areas of the following spectral ranges were determined: 3040-3000 cm^{-1} (=CH groups in lipid alkyl chains; CH), 3000-2800 cm^{-1} (CH2 and CH3 groups in lipid alkyl chains; LIP), 1772-1713 cm^{-1} (C=O moiety in fatty acids; FA), 1708-1480 cm^{-1} (Amide I and II bands of proteins; PRT), 1281-1191 cm^{-1} (phosphates; PH), and 1191-1134 cm^{-1} (COH groups in carbohydrates; COH). The following band area ratios were then calculated: LIP/TBM, FA/TBM, CH/TBM, PRT/TBM, PH/TBM, and COH/TBM. Each value was the ratio between the integrated area of the above defined spectral intervals and the integrated area of the whole spectrum (TBM). For each dietary group, the average absorbance spectrum was also calculated together with the corresponding standard deviation spectra (average absorbance spectrum ± standard deviation spectra).

Liver samples. From the middle part of each liver sample, three thin sections (10 μm thick) were cut at 100 μm intervals by using a cryostat MC400 (Histo-Line Laboratories, Pantigliate, Milano, Italy) and deposited onto CaF_2 optical windows (1 mm thick, 13 mm diameter). FTIR measurements were carried out by using a Bruker Invenio interferometer coupled with a Hyperion 3000 Vis-IR microscope and a bidimensional Focal Plane Array (FPA) detector (Bruker Optics GmbH). On each section, 2/3 IR maps were collected in transmission mode in the 4000-800 cm^{-1} spectral region; each IR map was a square of 164 μm per side and was formed by 4096 pixel/spectra (2.56 × 2.56 μm spatial resolution, 4 cm^{-1} spectral resolution, 128 scans). Before the acquisition of each IR map, a background spectrum was collected on a clean area of the CaF_2 optical window. Raw IR maps were corrected to avoid the contribution of atmospheric carbon dioxide and water vapor and then vector normalized to correct small differences in the thickness of the sample (OPUS 7.1).

From each IR map, false color images representing the topographical distribution of specific biocomponents were generated by integration under the following spectral ranges: 3000-2825 cm^{-1} (representative of lipids; LIP images), 1760-1725 cm^{-1} (representative of fatty acids; FA images), 1725-1480 cm^{-1} (representative of proteins; PRT images), 1280-1183 cm^{-1} (representative of phosphates; PH images), 1183-1136 cm^{-1} (representative of carbohydrates; COH images), and 1067-983 cm^{-1} (representative of glycogen; GLY images).

The univariate analysis of the biochemical composition of liver samples of all dietary groups was performed by calculating specific band area ratios representative of the relative amount of lipids (LIP/TBM), fatty acids (FA/TBM), proteins (PRT/TBM), phosphates (PH/TBM), carbohydrates (COH/TBM), and glycogen (GLY/TBM). Each value was the ratio between the integrated area of the above defined spectral intervals and the integrated area of the whole spectrum (TBM).

Microbiome

RNA extraction and cDNA synthesis. Extracted intestines (20 samples per tank, 60 samples per dietary treatment) were added with sterile physiological solution (0.85% NaCl, w/v) at a 1:10 ratio and homogenized for 3 min at 260 rpm in Stomacher apparatus (400 Circulator, International PBI, Milan, Italy). An aliquot (500 µL) of each 10^{-1} dilution was centrifuged for 10 min at 14,000 rpm to produce cell pellets subsequently protected by RNA later Stabilization Solution (Ambion, Foster City, CA, USA) and stored at -80 °C. Total microbial RNA was extracted from cell pellets by Quick-RNA Miniprep kit (Zymo Research, Irvine, CA, USA) following the manufacturer's instructions. The extracted RNA were checked for the quantity, purity and the absence of DNA contamination as previously described by Zarantoniello et al. [**19**] (Chapter 3.1). SensiFAST cDNA Synthesis Kit for RT-qPCR (Bioline, London, UK) was used for the synthesis of the cDNA starting from 10 µL each sample RNA.

16S rRNA amplicon target sequencing. V3-V4 region of the 16S rRNA gene was amplified via PCR as previously described by Klindworth et al. [**40**] using cDNA from each sample as a template. PCR products were prepared for the sequencing by MiSeq Illumina instrument (Illumina) with V3 chemistry as previously detailed by Zarantoniello et al. [**19**] (Chapter 3.1). Paired-end reads were assembled with FLASH [**41**] and quality filtered (at Phred < Q20) using QIIME 1.9.0 software and the pipeline recently described [**42**]. OTUs were clustered at 97% of similarity and taxonomy was assessed by Greengenes database v. 2013. OTU table was rarefied at the lowest number of sequence and display the higher taxonomy resolution.

Molecular analyses

RNA extraction and cDNA synthesis. Total RNA extractions from both liver and intestine samples from 5 different specimens from each tank (15 per dietary group) were performed using RNAzol RT reagent (Merck KGaA) according to Piccinetti et al [**43**]. Total RNA extracted was eluted in 40 µl of RNase-free water (Qiagen). Final RNA concentration was determined by the NanoPhotometer P-Class (Implen, München, Germany). RNA integrity was verified by GelRed™ staining of 28S and 18S ribosomal RNA bands on 1% agarose gel. RNA was stored at -80°C until use. Finally, 1 µg of total RNA were used for cDNA synthesis, employing the LunaScript RT SuperMix Kit (New England Biolabs, Ipswich, Massachusetts, USA) following the manufacturer's instructions.

Real-Time PCR. PCR were performed with SYBER green method in an iQ5 iCycler thermal cycler (Bio-Rad Laboratories) following Zarantoniello et al. [**19**] (Chapter 3.1). Relative quantification of the expression of genes involved in fish growth (insulin-like growth factor 1, *igf1*; insulin-like growth factor 2a, *igf2a;* myostatin, *mstnb*), stress response (glucocorticoid receptor, *nr3c1*; heat shock protein

70, *hsp70.1*), long-chain polyunsaturated fatty acids biosynthesis (fatty acid elongase 2, *elovl2*; fatty acid elongase 5, *elovl5;* fatty acid desaturase 2, *fads2*), appetite response (ghrelin, *ghrl*; cannabinoid receptor 1, *cnr1*; leptin a, *lepa*), immune response (interleukin 1β, *il1b*; interleukin 10, *il10*; tumor necrosis factor a, *tnfa*) and enzymatic hydrolysis of chitin (chitinase 2, *chia.2*; chitinase 3, *chia.3*) was performed. Actin-related protein 2/3 complex, subunit 1A (*arpc1a*) and ribosomal protein, large, 13 (*rpl13*) were used as internal standards in each sample in order to standardize the results by eliminating variation in mRNA and cDNA quantity and quality. No amplification products were observed in negative controls and no primer-dimer formations were observed in the control templates. Amplification products were sequenced, and homology was verified. The data obtained were analysed using the iQ5 optical system software version 2.0 (Bio-Rad) including GeneEx Macro iQ5 Conversion and genex Macro iQ5 files. Primer sequences were designed using Primer3 (210 v. 0.4.0) starting from zebrafish sequences available in ZFIN. Primer sequences used were reported in Table 2.

Table 2. Sequences, Zebrafish Information Network (ZFIN) IDs, and annealing temperature (A.T.) of the primers used for the present study.

Gene	Forward Primer (5'-3')	Reverse Primer (5'-3')	ZFIN IDs	A. T. (°C)
igf1	GGCAAATCTCCACGATCTCTAC	CGGTTTCTCTTGTCTCTCTCAG	ZDB-GENE-010607-2	53
igf2a	GAGTCCCATCCATTCTGTTG	GTGGATTGGGGTTTGATGTG	ZDB-GENE-991111-3	59
mstnb	GGACTGGACTGCGATGAG	GATGGGTGTGGGGATACTTC	ZDB-GENE-990415-165	58
nr3c1	AGACCTTGGTCCCCTTCACT	CGCCTTTAATCATGGGAGAA	ZDB-GENE-050522-503	58
hsp70.1	TGTTCAGTTCTCTGCCGTTG	AAAGCACTGAGGGACGCTAA	ZDB-GENE-990415-91	58
elovl2	CACTGGACGAAGTTGGTGAA	GTTGAGGACACACCACCAGA	ZDB-GENE-011212-1	60
elovl5	TGGATGGGACCGAAATACAT	GTCTCCTCCACTGTGGGTGT	ZDB-GENE-040407-2	60
fads2	CATCACGCTAAACCCAACA	GGGAGGACCAATGAAGAAGA	ZDB-GENE-011212-1	60
ghrl	CAGCATGTTTCTGCTCCTGTG	TCTTCTGCCCACTCTTGGTG	ZDB-GENE-070622-2	58
cnr1	AGCAAAAGGAGCAACAGGCA	GTTGGTCTGGTACTTTCACTTGAC	ZDB-GENE-040312-3	60
lepa	CTCCAGTGACGAAGGCAACTT	GGGAAGGAGCCGGAAATGT	ZDB-GENE-081001-1	58
il1b	GCTGGGGATGTGGACTTC	GTGGATTGGGGTTTGATGTG	ZDB-GENE-040702-2	54
il10	ATTTGTGGAGGGCTTTCCTT	AGAGCTGTTGGCAGAATGGT	ZDB-GENE-051111-1	56
tnfa	TTGTGGTGGGGTTTGATG	TTGGGGCATTTTATTTTGTAAG	ZDB-GENE-050317-1	53
chia.2	GGTGCTCTGCCACCTTGCCTT	GGCATGGTTGATCATGGCGAAAGC	ZDB-GENE-040426-2014	64
chia.3	TCGACCCTTACCTTTGCACACACCT	ACACCATGATGGAGAACTGTGCCGA	ZDB-GENE-040426-2891	65
arpc1a	CTGAACATCTCGCCCTTCTC	TAGCCGATCTGCAGACACAC	ZDB-GENE-040116-1	60
rpl13	TCTGGAGGACTGTAAGAGGTATGC	AGACGCACAATCTTGAGAGCAG	ZDB-GENE-031007-1	59

Behavioural tests

Open field test. The open-field test used in the present study followed the procedure commonly adopted to study fish [**44**] and to assess effects of diets [**45**]. Each individual (16 fish per dietary

group; 80 fish overall) was placed in an unfamiliar, empty arena (40 × 40 cm, 10 cm of water) for a brief period (15 min). A light source [Light-emitting diodes (LED), warm white; Superlight Technology Co. Ltd., Shenzhen, China] was placed 1 m above the area. The spontaneous behaviour of the subject was recorded by means of video tracking system equipped with an IR-sensitive camera (Monochrome GigE camera, Basler, Ahrensburg, Germany; resolution: 1,280 × 1,024). Using Ethovision 11 software (Noldus Information Technology, Wageningen, NL), two variables were analysed: (i) the activity of the subject (measured as distance travelled) and (ii) the time spent in the centre of the open field (1 body length from the edges). Bolder, more explorative, and less anxious fish were expected to travel greater distances in the open field and spend more time in the central area.

Photic behavioural entrainment test. The photic behavioural entrainment test was performed following a standard procedure [**46**]. Because the test required extended recordings, zebrafish were tested in groups formed by four individuals to avoid social isolation. The subjects were different from those used in the previous behavioural test, and therefore data of the two tests were independent. Because the previous behavioural test did not provide evidence of different behaviours between the dietary groups, here only fish from the Hi0 group (n groups = 4, n zebrafish = 16) and from the dietary treatment with higher BSF prepupae meal inclusion (Hi100 diet; n groups = 4, n zebrafish = 16) were compared. Each group was kept in a square arena (20 × 20 cm) and exposed to a 12:12 light–dark cycle. For light sources, LED (Superlight Technology Co. Ltd) were used. Irradiance was measured with a radiometer (DO9721, Probe LP9021 RAD, spectral range 400-950 nm, DeltaOHM, Padova, Italy) and set at 0.6 W/m^2. The temperature was held constant at 28 °C by means of a thermostatically controlled heater. Zebrafish locomotor activity was recorded for 70 consecutive hours and then analysed by Ethovision 11. The IR-sensitive camera was set to 5 frames per second. Locomotor activity of each group was calculated as the total distance moved during a 6 min time window (700 observations per subject). A minimal distance moved of 2 mm was used. In case of normal photic behavioural entrainment, zebrafish were expected to show markedly diurnal activity [**46**].

Statistical analyses

All data (except for microbiome) were analysed by one-way ANOVA, with diet as the explanatory variable. All ANOVA tests were followed by Tukey's post-hoc tests. The statistical software package Prism5 (GraphPad Software) was used. Significance was set at $p < 0.05$ and all results are presented as mean ± SD. As regards microbiome, bioinformatics analysis were performed as described by Osimani et al. [**18**] and Zarantoniello et al. [**19**] (Chapter 3.1).

Results

Growth and survival

Considering specific growth rate (SGR %; Fig. 1), only Hi75 and Hi100 groups (13.0 ± 0.4 and 13.2 ± 0.4 %, respectively) showed significantly higher values than Hi0 (12.3 ± 0.6 %). No significant differences were evident among Hi0, Hi25, and Hi50 groups (12.7 ± 0.8 and 12.7 ± 0.9 % for Hi25 and Hi50, respectively). As regards survival, no significant differences were observed among the experimental groups.

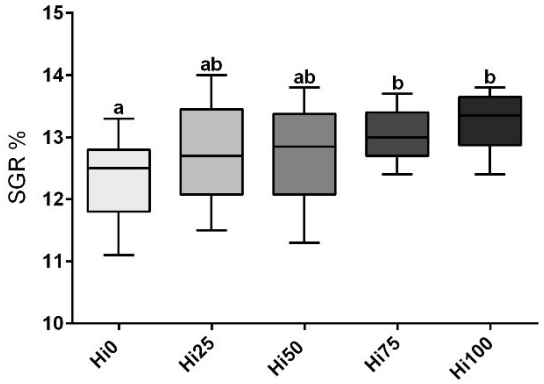


Figure 1. Specific growth rate (% weight growth/day) of zebrafish. Boxplots show minimum and maximum (whiskers), first quartile, median and third quartile (box). Different letters denote statistically significant differences among experimental groups ($p < 0.05$). Zebrafish fed diets including 0, 25, 50, 75 and 100 % of BSF prepupae meal respect to FM (Hi0, Hi25, Hi50, Hi75 and Hi100, respectively).

Fatty acids content and composition

Diets. Results obtained from the experimental diets were previously reported in Zarantoniello et al. [**19**] (chapter 3.1). Briefly, as reported in Figure 2a, BSF-based diets showed significantly higher percentages of SFA and significantly lower percentages of monounsaturated fatty acids (MUFA) and PUFA with respect to Hi0 diet. However, increasing BSF full-fat prepupae meal inclusion levels in the diets resulted in a PUFA increase from Hi25 to Hi100. Increasing inclusion levels of BSF full-fat prepupae meal in the diets resulted in a significant decrease of n3 percentages and a parallel significant increase in n6 percentages. Consequently, the n6/n3 ratio showed significant differences among experimental diets, increasing from Hi0 to Hi100 diet (Fig. 2b).

Zebrafish. Figure 2c reports the percentages of the different fatty acid classes in zebrafish fed the different diets. The increasing inclusion levels of enriched BSF prepupae meal resulted in: (i) a significant increase in SFA (28.0 ± 0.7, 30.0 ± 0.5, 35.2 ± 0.6, 36.6 ± 0.8 and 39.2 ± 0.4 % for Hi0, Hi25, Hi50, Hi75 and Hi100, respectively) and n6 (12.9 ± 0.2, 14.1 ± 0.2, 15.9 ± 0.2, 19.2 ± 0.2 and 19.5 ± 0.2 % for Hi0, Hi25, Hi50, Hi75 and Hi100, respectively) percentages; (ii) a significant decrease in both PUFA (42.7 ± 0.7, 40.5 ± 0.8, 35.6 ± 0.8, 35.0 ± 1.1 and 31.9 ± 0.8 % for Hi0, Hi25,

Hi50, Hi75 and Hi100, respectively) and n3 (29.3 ± 0.7, 26.5 ± 0.8, 19.7 ± 0.8, 15.7 ± 1.1 and 12.3 ± 0.7 % for Hi0, Hi25, Hi50, Hi75 and Hi100, respectively) percentages. Regarding MUFA content, groups fed with the higher BSF prepupae meal inclusion levels showed significantly lower percentages (28.4 ± 0.3 and 28.8 ± 0.2 % for Hi75 and Hi100, respectively) with respect to Hi0. Hi0, Hi25 and Hi50 (29.3 ± 0.4, 29.5 ± 0.3 and 29.2 ± 0.3 %, respectively) did not show significant differences among them. Considering n9 content, all the groups fed BSF-based diets were characterized by significantly lower percentages (16.8 ± 0.1, 16.8 ± 0.2, 16.8 ± 0.2 and 16.6 ± 0.2 % for Hi25, Hi50, Hi75 and Hi00, respectively) than Hi0 (17.1 ± 0.2 %). Finally, the n6/n3 ratio (Fig. 2d) increased according to the increasing dietary BSF prepupae meal inclusion level (0.43 ± 0.01, 0.53 ± 0.02, 0.81 ± 0.03, 1.22 ± 0.09 and 1.60 ± 0.10 for Hi0, Hi25, Hi50, Hi75 and Hi100, respectively).

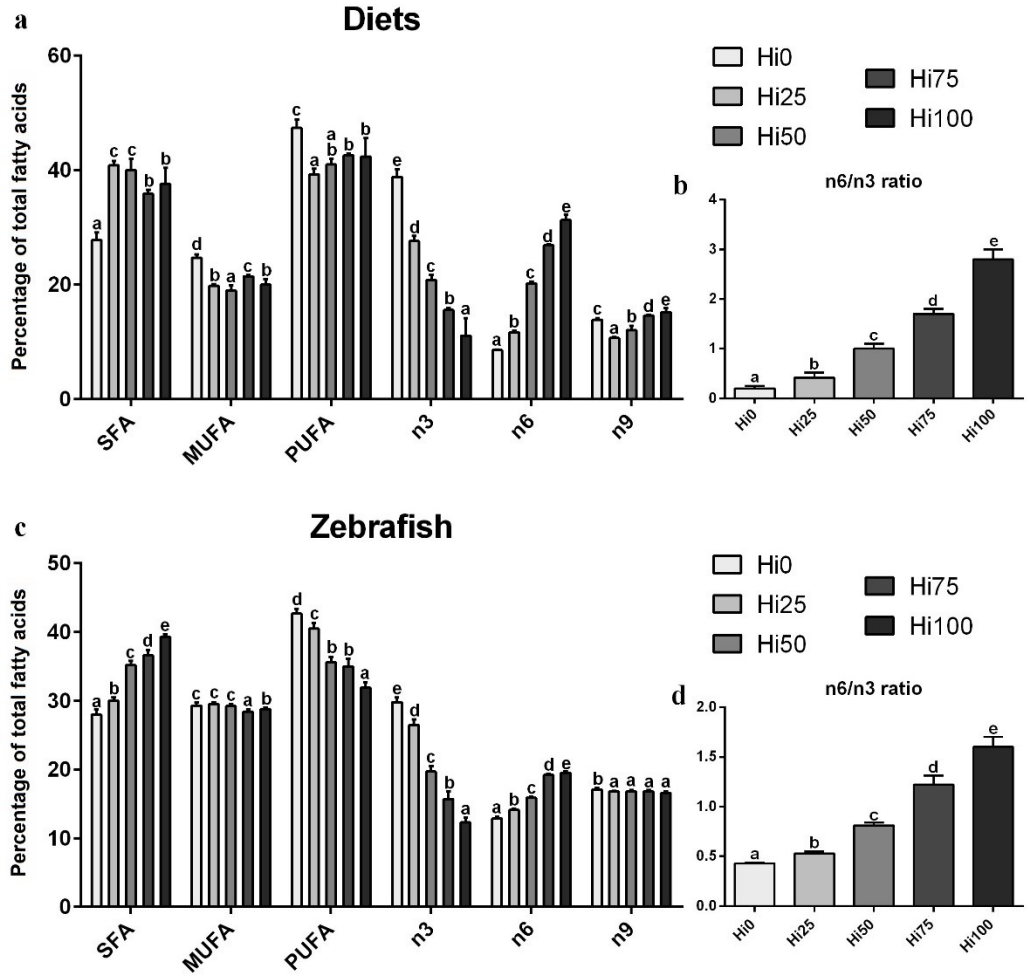


Figure 2. Content of SFA, MUFA and PUFA (as % of total fatty acids) and contribution of omega 3 (n3), omega 6 (n6) and omega 9 (n9) fatty acids to lipid profile. (**a**,**b**) experimental diets; (**c**,**d**) zebrafish. Different letters indicate statistically significant differences among experimental groups compared within the same fatty acids class ($p < 0.05$). Values are presented as mean ± SD (n = 3 for experimental diets; n = 15 for zebrafish). Zebrafish fed diets including 0, 25, 50, 75 and 100 % of BSF prepupae meal respect to FM (Hi0, Hi25, Hi50, Hi75 and Hi100, respectively).

Table 3. Zebrafish fatty acids composition (as % of total fatty acids).

	Hi0	Hi25	Hi50	Hi75	Hi100
10:0	0.02±0.01	0.04±0.01	0.12±0.01	0.13±0.01	0.15±0.02
12:0	0.27±0.01 [a]	3.0±0.3 [b]	6.2±0.2 [c]	7.2±0.3 [d]	8.3±0.5 [e]
13:0	0.04±0.01	0.05±0.01	0.07±0.01	0.08±0.02	0.08±0.02
14:0	4.3±0.3 [a]	4.5±0.3 [a]	5.1±0.3 [bc]	4.9±0.1 [b]	5.2±0.4 [c]
14:1n5	0.27±0.02	0.33±0.05	0.40±0.03	0.45±0.07	0.59±0.09
15:0	0.78±0.03	0.79±0.02	0.81±0.06	0.84±0.13	0.84±0.16
16:0	16.8±1.5 [b]	15.9±0.3 [a]	16.9±0.9 [b]	17.9±1.1[c]	18.2±0.8 [c]
16:1n9	0.76±0.05	0.78±0.03	0.90±0.17	0.89±0.12	0.87±0.07
16:1n7	7.7±0.2 [b]	7.8±0.3 [b]	7.8±0.1 [b]	7.2±0.3 [a]	7.1±0.1 [a]
17:0	0.81±0.03	0.84±0.1	0.75±0.1	0.81±0.1	0.90±0.1
17:1n7	0.81±0.12 [a]	1.1±0.04 [b]	1.2±0.04 [c]	1.4±0.11 [d]	1.6±0.02 [e]
18:0	4.8±0.8 [a]	4.7±0.5 [a]	5.0±1.0 [a]	4.7±0.6 [a]	5.4±1.1 [a]
18:1n9	14.6±0.8 [a]	14.7±0.5 [a]	15.1±0.1 [a]	15.3±0.9 [a]	15.4±1.4 [a]
18:1n7	3.4±0.2 [c]	3.5±0.2 [c]	3.0±0.2 [b]	2.7±0.1 [a]	2.9±0.1 [b]
18:2n6	10.7±0.6 [a]	11.3±0.2 [b]	12.9±0.1 [c]	15.7±1.0 [e]	15.2±0.6 [d]
18:3n6	0.33±0.02	0.38±0.02	0.40±0.04	0.58±0.06	0.52±0.03
18:3n3	2.2±0.2 [c]	2.2±0.1 [c]	2.0±0.2 [ab]	2.1±0.3 [bc]	1.8±0.2 [a]
20:0	0.18±0.01	0.16±0.01	0.18±0.03	0.19±0.02	0.18±0.04
20:1n9	1.0±0.04 [e]	0.83±0.06 [d]	0.56±0.03 [c]	0.43±0.05 [b]	0.33±0.02 [a]
20:2n6	0.32±0.03	0.32±0.02	0.32±0.05	0.32±0.01	0.44±0.06
20:3n6	0.38±0.04 [a]	0.50±0.02 [b]	0.68±0.03 [c]	0.85±0.10 [d]	1.2±0.1 [e]
20:4n6	1.2±0.1 [a]	1.5±0.1 [b]	1.6±0.1 [b]	1.8±0.1 [c]	2.2±0.1 [d]
20:3n3	0.18±0.02	0.16±0.01	0.13±0.02	0.11±0.02	0.13±0.04
20:5n3	10.7±0.7 [e]	9.5±0.8 [d]	6.1±0.8 [c]	3.6±0.4 [b]	2.0±0.5 [a]
22:1n9	0.51±0.1	0.36±0.02	0.18±0.01	0.11±0.04	0.03±0.01
22:6n3	16.8±1.8 [e]	14.7±1.1 [d]	11.5±0.8 [c]	10.0±0.2 [b]	8.4±0.7 [a]
24:1n9	0.21±0.03	0.13±0.01	0.04±0.01	0.02±0.01	0.01±0.01
DHA/EPA	1.6±0.1 [a]	1.6±0.1[a]	1.9±0.1 [b]	2.8±0.3 [c]	4.4±0.4 [d]

Means within rows bearing different letters are significantly different ($p < 0.05$). Statistical analysis was performed only for fatty acids > 1%. Fatty acids with a percentage < 1% were excluded from statistical analyses because their concentrations were close to the limit of detection. Zebrafish fed diets including 0, 25, 50, 75 and 100 % of BSF prepupae meal respect to FM (Hi0, Hi25, Hi50, Hi75 and Hi100, respectively)

As concern zebrafish fatty acids composition (Table 3), the most represented SFA in all the experimental groups were palmitic (16:0) and stearic (18:0) acids. Furthermore, the percentage of major (> 1%) SFA (18:0 excluded) significantly increased according to the increasing BSF prepupae meal inclusion. In particular, the content of lauric acid (12:0) increased up to ~ 30-folds from Hi0 to Hi100 group. Considering MUFA, the predominant fatty acid in all the experimental groups was oleic acid (18:1n9) which did not show significant differences among the experimental groups. Furthermore, the other major MUFA such as palmitoleic (16:1n7) and vaccenic (18:1n7) acids

showed a significant decrease in fish fed diets with increasing BSF prepupae meal inclusion levels respect to FM from 50 (Hi50) to 100% (Hi100). Linoleic (18:2n6) and docosahexaenoic (22:6n3; DHA) acids were the most abundant PUFA in all the dietary treatments. Linoleic and arachidonic (20:4n6) acids showed a significant increase in fish fed diets with increasing BSF prepupae meal inclusion levels. A significant decrease in eicosapentaenoic acid (20:5n3; EPA) percentage was detected in fish fed diets with increasing BSF prepupae meal inclusion levels, whereas as regards DHA, a similar, but milder trend was observed respect to EPA. Consequently, the DHA/EPA ratio significantly increased with the increasing dietary BSF prepupae meal inclusion levels respect to FM.

Histology

Histological analyses were performed in order to detect possible inflammatory events in fish intestine and to evaluate lipid accumulation or steatosis in liver. As concerns intestine (Fig. 3a-j), no morphological alterations or signs of inflammation were evident in all the experimental groups (Fig. 3a,f: Hi0; Fig. 3b-e and g-j: Hi groups).

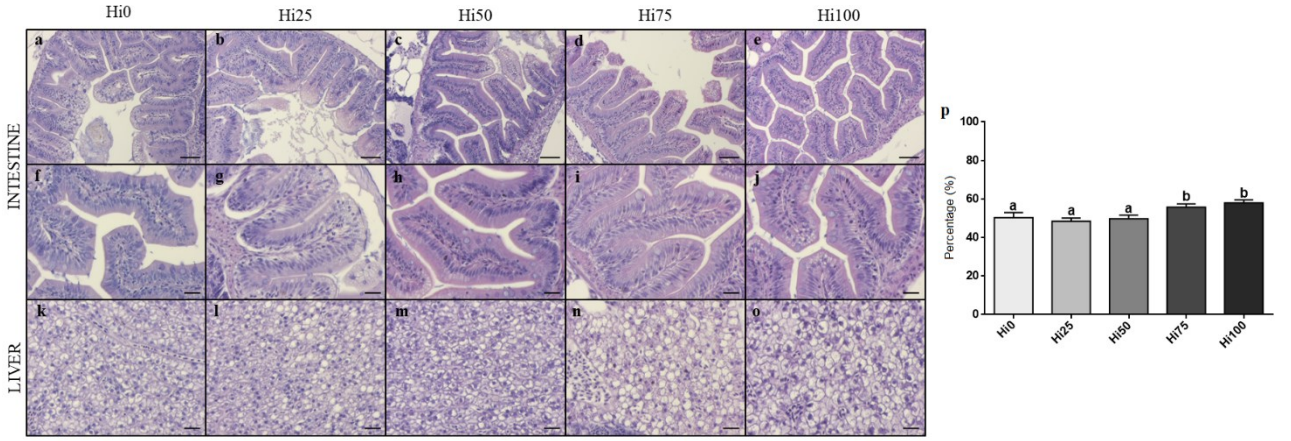


Figure 3. Zebrafish intestine and liver histology and percentage of fat fraction (PFF) in liver tissue. (**a-j**) intestine; (**k-o**) liver; (**p**) PFF. Scale bars: (**a-e**) 50 μm; (**f-j**) 20 μm; (**k-o**) 10 μm. For PFF, values are shown as mean ± SD (n = 15). Different letters indicate statistically differences among the experimental groups ($p < 0.05$). Zebrafish fed diets including 0, 25, 50, 75 and 100 % of BSF prepupae meal respect to FM (Hi0, Hi25, Hi50, Hi75 and Hi100, respectively).

Considering liver, results evidenced a variable degree of lipid accumulation in all the experimental groups (Fig. 3k-o). Hi0, Hi25 and Hi50 groups were characterized by a modest fat liver parenchyma with a diffuse presence of hepatocytes with cytoplasm filled of fat, interspersed with normal hepatocytes. Conversely, Hi75 and Hi100 groups showed a severe degree of steatosis with swollen hepatocytes and abundant intracytoplasmic lipid accumulation (Fig. 3n,o). These results were confirmed by the quantification of the percentage of fat fraction (PFF; Fig. 3p) on liver sections. In particular, no significant differences were evident among Hi0, Hi25 and Hi50 groups (50.3 ± 2.6, 48.3 ± 1.7, 50.0 ± 2.0 % for Hi0, Hi25 and Hi50, respectively), while Hi75 and Hi100 groups (55.6

± 1.7 and 58.0 ± 1.4 % for Hi75 and Hi100, respectively) showed significantly higher values respect to the other experimental groups.

FTIR analysis

Brain samples. The average spectra of brain samples of all dietary groups are reported in Figure 4a. The most significant IR bands (reported in the upper part of Fig. 4a), are listed below, together with the position (wavenumbers) and the biological meaning: ~ 3015 cm^{-1} (=CH moieties in lipid alkyl chains); ~ 2922 cm^{-1} and ~ 2852 cm^{-1} (CH2 groups in lipid alkyl chains); ~ 1744 cm^{-1} (C=O moiety in lipids and fatty acids); ~ 1645 cm^{-1} and ~ 1540 cm^{-1} (Amide I and II bands of proteins, respectively); ~ 1467 cm^{-1} (CH2 and CH3 groups in lipids and proteins side chains); ~ 1398 cm^{-1} (COO^- groups in amino acids); ~ 1235 cm^{-1} (phosphate groups); ~ 1173 cm^{-1} (glycosylated compounds); ~ 1062 cm^{-1} (carbohydrates).

The univariate analysis of the biochemical composition of brain samples of all dietary groups (Fig. 4b) was performed by calculating specific band area ratios representative of the relative amounts of lipids (LIP/TBM), fatty acids (FA/TBM), proteins (PRT/TBM), carbohydrates (COH/TBM), phosphates (PH/TBM) and of the degree of unsaturation in lipid alkyl chains (CH/TBM). Statistically significant lower amounts of total lipids (LIP/TBM) and significant higher amounts of proteins (PRT/TBM) were detected in Hi75 and Hi100 brain samples compared to the other experimental groups. A significant decrement in total fatty acids and unsaturated ones (FA/TBM and CH/TBM, respectively) and carbohydrates (COH/TBM) was detected in all the groups fed BSF-based diets. No significant differences were observed among the experimental groups as regards phosphate groups (PH/TBM).

Liver samples. The hyperspectral imaging analysis of representative liver sections of zebrafish from the dietary groups is shown in Fig. 5a. The generated false colour images displayed the topographical distribution of lipids (LIP), fatty acids (FA), proteins (PRT), phosphates (PH), carbohydrates (COH) and glycogen (GLY). By considering the different scales adopted for each macromolecule, livers from Hi0 group were characterized by a higher amount of proteins (PRT images, numerical scale 0-10) with respect to lipids (LIP images, numerical scale 0-5) and glycogen (GLY images, numerical scale 0-3). As regards the effects of the different diets, in all dietary groups containing increasing inclusion levels of BSF prepupae meal, an increment of total lipids (LIP images) and fatty acids (FA images) and a decrement of proteins (PRT images) and phosphate groups (PH images) were observed. Minor amounts of total carbohydrates and glycogen were also detected in Hi25, Hi50 and Hi75 groups respect to Hi0, while Hi100 samples showed the highest levels.

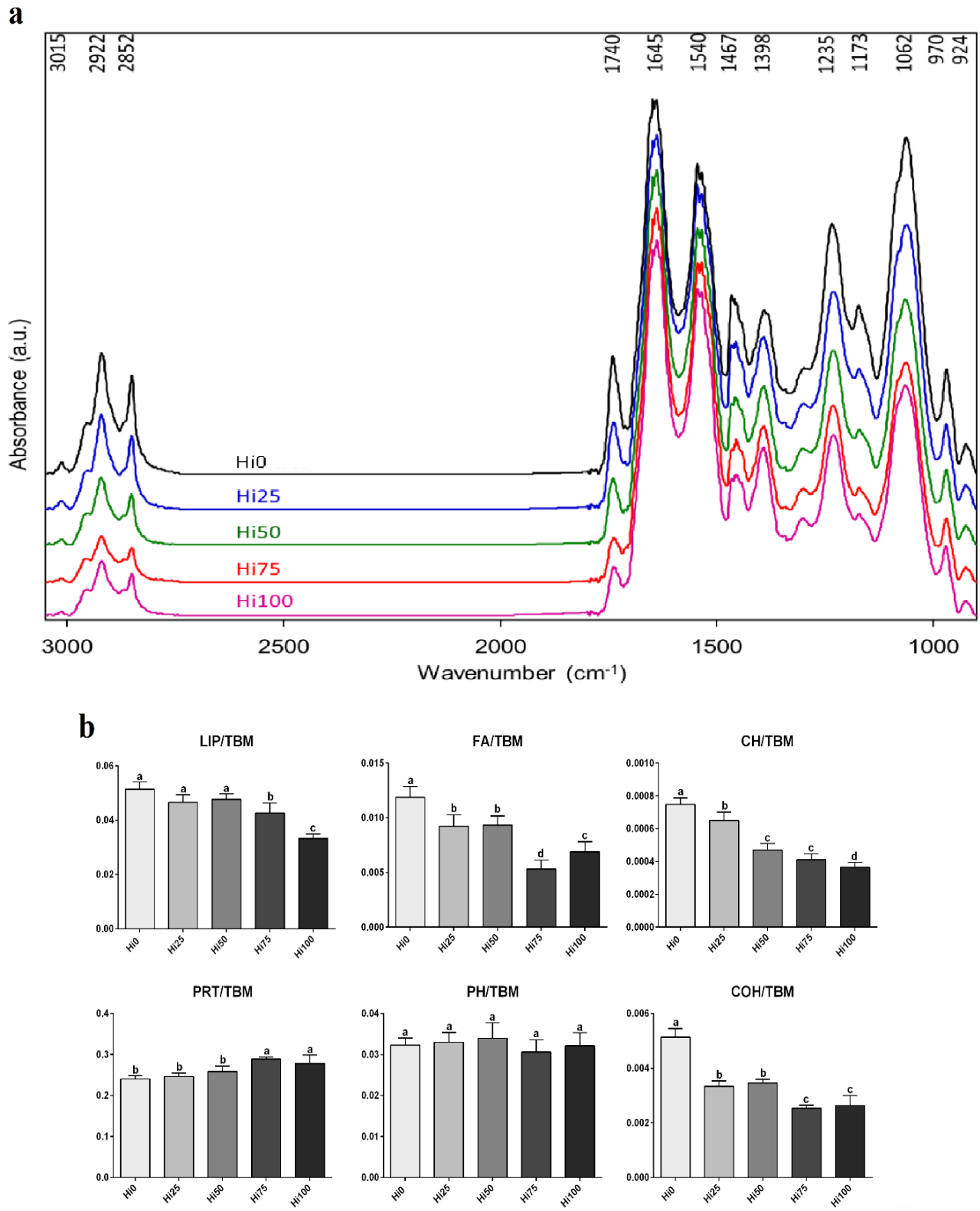


Figure 4. (**a**) Average absorbance spectra and (**b**) biochemical composition of zebrafish brain samples from the dietary groups. (**a**) Spectra are reported in the 3050-900 cm^{-1} spectral range and are shifted along the y-axis for a better understanding. The wavenumbers of the most significant bands are reported in the upper part. (**b**) Univariate analysis of the following band area ratios: LIP/TBM (relative amount of total lipids); FA/TBM (relative amount of fatty acids); CH/TBM (degree of unsaturation in lipid alkyl chains); PRT/TBM (relative amount of total proteins); PH/TBM (relative amount of phosphate groups), and COH/TBM (relative amount of carbohydrates). Data are reported as mean ± SD (n = 15). Different letters indicate statistically differences among the experimental groups ($p < 0.05$). Zebrafish fed diets including 0, 25, 50, 75 and 100 % of BSF prepupae meal respect to FM (Hi0, Hi25, Hi50, Hi75 and Hi100, respectively).

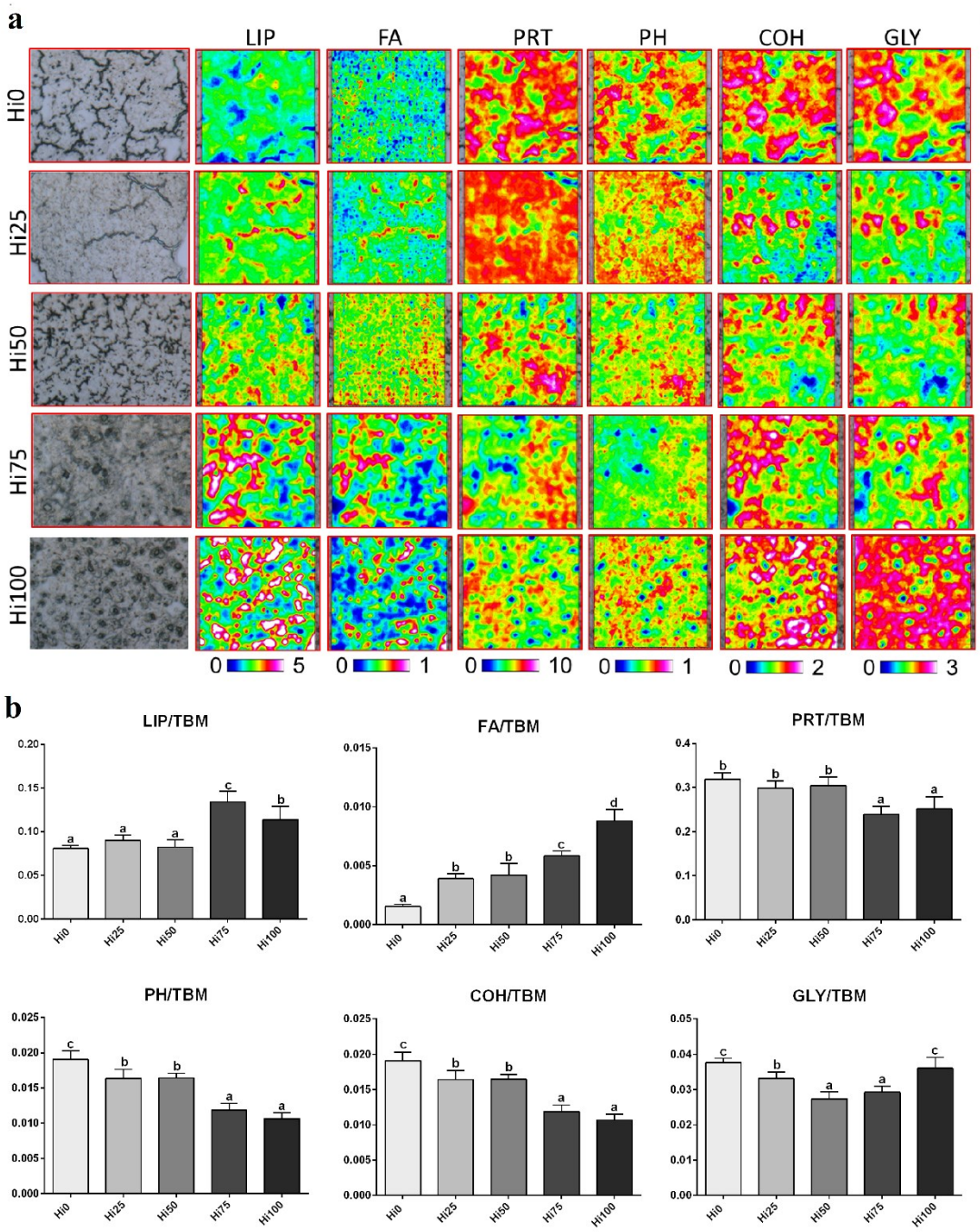


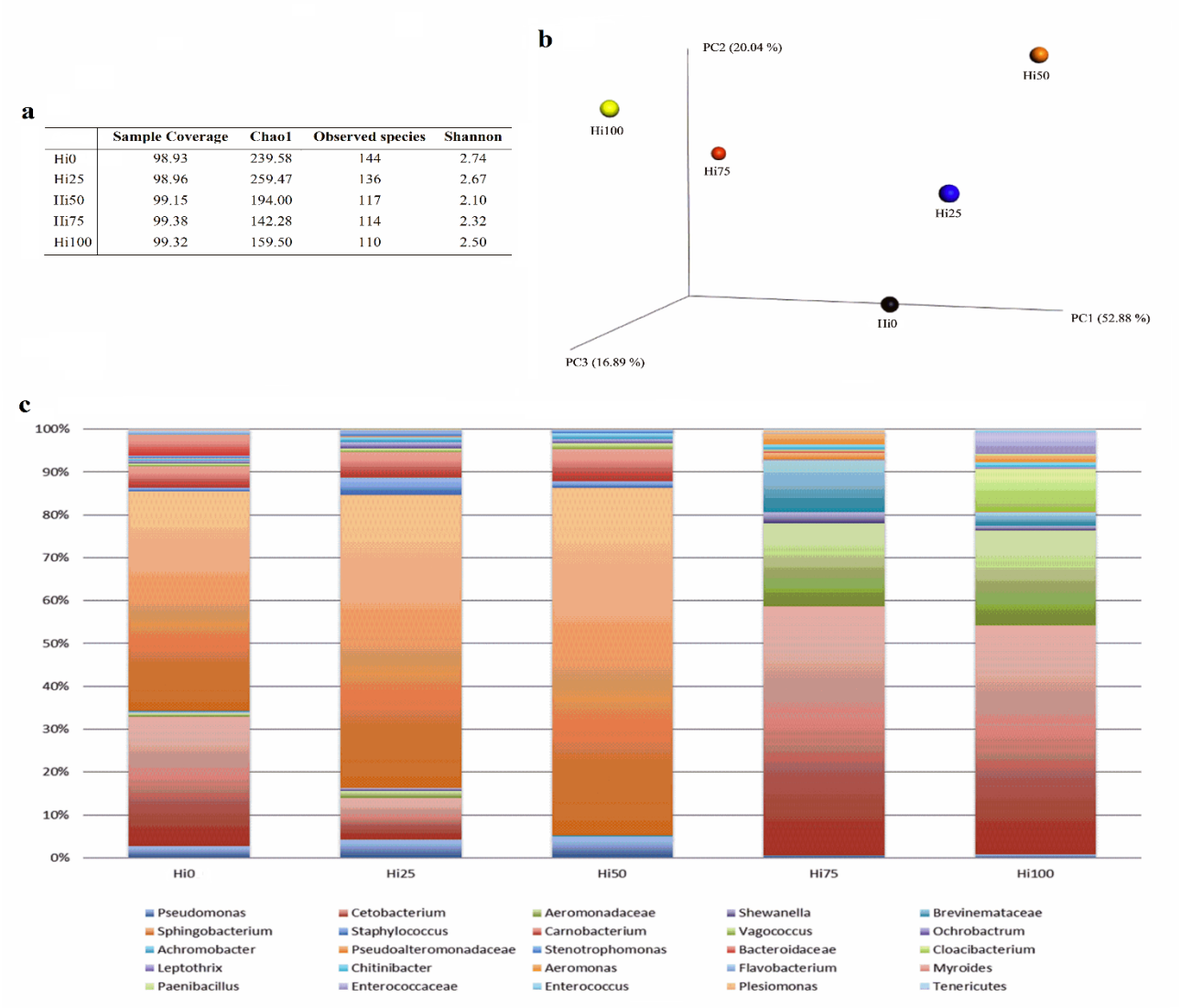
Figure 5. (**a**) Microphotographs, hyperspectral analysis, and (**b**) biochemical composition of representative liver sections from all dietary groups. (**a**) False colour images showing the topographical distribution of lipids (LIP), fatty acids (FA), proteins (PRT), phosphate groups (PH), carbohydrates (COH), and glycogen (GLY). (**b**) Univariate analysis of the following band area ratios: LIP/TBM (relative amount of total lipids); FA/ TBM (relative amount of fatty acids); PRT/TBM (relative amount of total proteins); PH/TBM (relative amount of phosphate groups); COH/TBM (relative amount of total carbohydrates), and GLY/TBM (relative amount of glycogen). Data are reported as mean ± SD (n = 15). Different letters indicate statistically differences among the experimental groups ($p < 0.05$). Zebrafish fed diets including 0, 25, 50, 75 and 100 % of BSF prepupae meal respect to FM (Hi0, Hi25, Hi50, Hi75 and Hi100, respectively).

The biochemical composition of liver samples of all dietary groups (Fig. 5b) was then investigated by the univariate analysis of specific band area ratios, representative of the relative amount of lipids (LIP/TBM), fatty acids (FA/TBM), proteins (PRT/TBM), phosphates (PH/TBM), carbohydrates (COH/TBM), and glycogen (GLY/TBM). Significantly higher amounts of total lipids (LIP/TBM) and

significantly lower amounts of total proteins (PRT/TBM) were detected only in Hi75 and Hi100 liver samples. An increasing trend in fatty acids amount (FA/TBM) was detected in all livers samples of fish fed diets with increasing BSF prepupae meal inclusion levels respect to FM, together with a significant decrease of both carbohydrates (COH/TBM) and phosphates (PH/TBM). Finally, significantly lower levels of glycogen (GLY/TBM) were found in Hi50 and Hi75 groups.

Microbiome

After sequencing and quality filtering, 51,554 reads were used for the downstream analysis with a median value of 10,572 ± 2411 reads/sample.



	Sample Coverage	Chao1	Observed species	Shannon
Hi0	98.93	239.58	144	2.74
Hi25	98.96	259.47	136	2.67
IIi50	99.15	194.00	117	2.10
IIi75	99.38	142.28	114	2.32
Hi100	99.32	159.50	110	2.50

Figure 6. Zebrafish microbiome. (**a**) Number of sequences analysed, observed diversity and sample coverage for 16 s rrna amplicons acquired from zebrafish gut samples. (**b**) Principal coordinates analysis of weighted UniFrac distances for 16S rRNA gene sequence data assembled as a function of the dietary BSF prepupae meal inclusion level respect to FM. The first component (PC1) accounts for the 52.88% of the variance, the second one (PC2) for the 20.04% and the third one (PC3) for the 16.89%. (**c**) Frequency of the major taxonomic groups identified by sequencing. Only OTUs with an incidence > 0.2% are shown. Zebrafish fed diets including 0, 25, 50, 75 and 100 % of BSF prepupae meal respect to FM (Hi0, Hi25, Hi50, Hi75 and Hi100, respectively).

The estimated sample coverage indicated that there was a satisfactory coverage of all the samples (median value of 99%) and by comparing the alpha-diversity value a reduction both in number of species and in the chao1 index was correlated with the increase of BSF prepupae meal inclusion level respect to FM in the diets (Fig. 6a).

By plotting the Principal Coordinate Analysis (PCoA) of the UNIFRAC distance matrix (Fig. 6b) a separation of the samples as functions of the amount of BSF prepupae meal inclusion level in the diet was observed. Figure 6c displays the microbiota composition distribution across samples. In detail, *Sphingobacterium* dominated the microbiota in Hi0, Hi25 and Hi50 fish, whereas the same genus was absent in Hi75 and Hi100 groups. In the Hi75 and Hi100 fish gut, *Cetobacterium*, Aeromonadaceae, and Brevinemataceae reached 50, 20 and 10 % of the relative abundance, respectively. The presence of *Cloacibacterium*, Enterococcaceae, Brevinemataceae, *Aeromonas* and *Shewanella* was also observed in Hi75 and Hi100 fish groups (Fig. 6c).

Real-time PCR results

Growth factors. As reported in Fig. 7a, *igf1* gene expression showed a slight downregulation from Hi0 to Hi100 group. Considering *igf2a* gene expression (Fig. 7b), all the experimental groups fed on BSF-based diets were characterized by a significant downregulation with respect to Hi0 group. An opposite trend was observed for *mstnb* gene expression (Fig. 7c) that was significantly upregulated particularly when considering Hi75 and Hi100 *vs* Hi0 groups.

Stress response. As regards *nr3c1* gene expression (Fig. 7d), all the experimental groups fed on BSF-based diets were characterized by a significant upregulation with respect to Hi0. Similarly, considering *hsp70.1* gene expression (Fig. 7e), Hi50, Hi75 and Hi100 groups showed a significant upregulation respect to Hi0 and Hi25 groups, which did not present significant differences between them.

Lipid metabolism. As concerns *elovl5* gene expression (Fig. 7f), no significant differences were evident among Hi0, Hi25 and Hi50 groups, while Hi75 and Hi100 groups showed a significant upregulation respect to the other experimental groups. Differently, considering *elovl2* and *fads2* gene expression (Fig. 7g,h) no significant differences were detected among the experimental groups.

Appetite. The experimental groups fed on BSF-based diets showed a significant dose-dependent increase in *ghrl* gene expression (Fig. 7i). Hi0 group presented a significant *ghrl* upregulation than Hi25 group and a significant *ghrl* downregulation than Hi100 group. Considering *cnr1* gene expression (Fig. 7j), no significant differences were detected among Hi0, Hi25 and Hi50 group. Differently, Hi75 and Hi100 groups showed a significant *cnr1* upregulation respect to the other groups (with the exception of Hi0 and Hi75 group which did not evidence significant

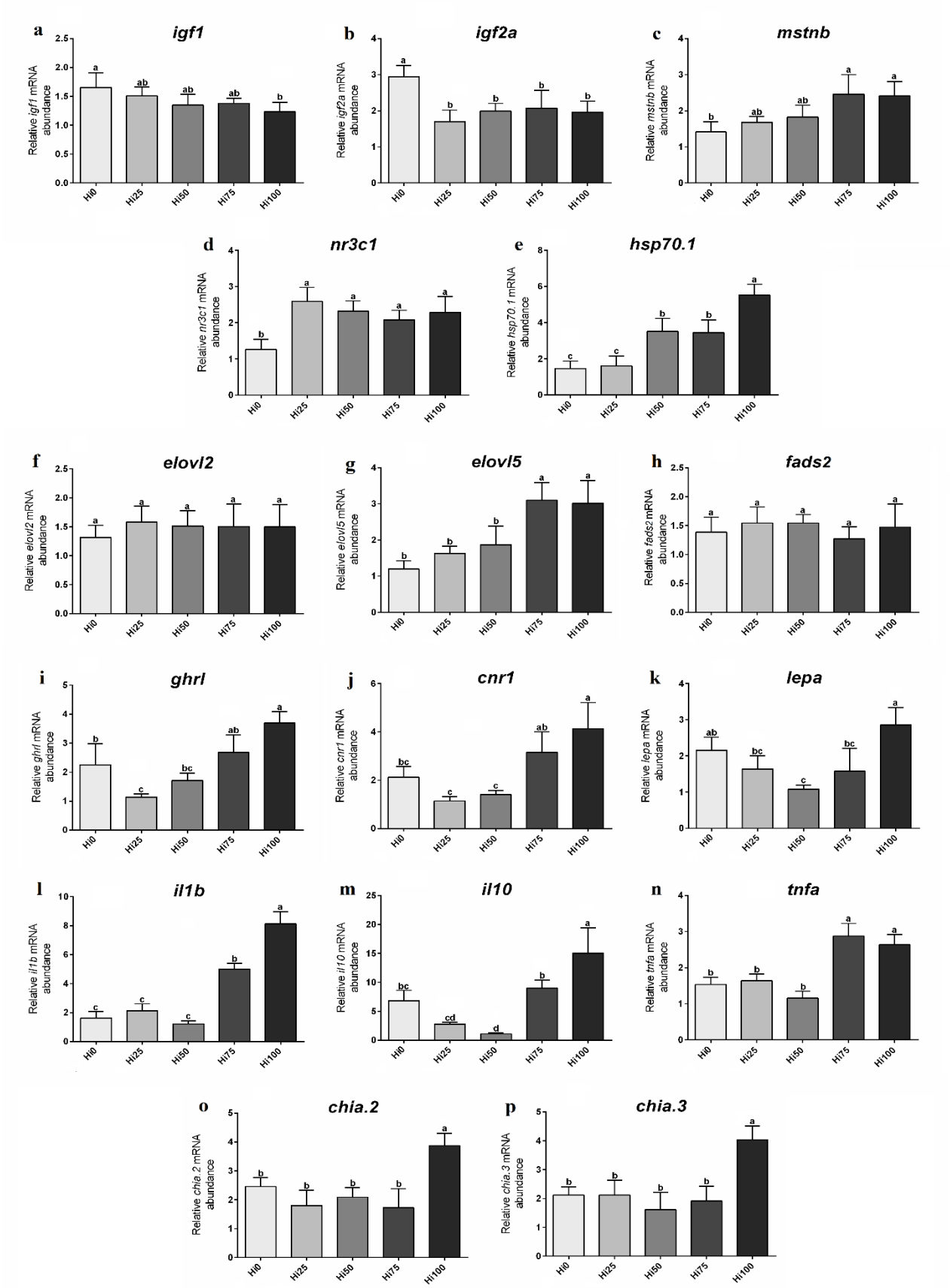


Figure 7. Relative mRNA abundance of genes analysed in zebrafish. (**a**) *igf1*, (**b**) *igf2a*, (**c**) *mstnb,* (**d**) *nr3c1*, (**e**) *hsp70.1,* (**f**) *elovl2*, (**g**) *elovl5* and (**h**) *fads2* were analysed in liver samples; (**i**) *ghrl*, (**j**) *cnr1*, (**k**) *lepa,* (**l**) *il1b*, (**m**) *il10*, (**n**) *tnfa,* (**o**) *chia.2* and (**p**) *chia.3* were analysed in intestine samples. Different letters specify statistically significant differences among groups ($p < 0.05$). Values are showed as mean ± SD (n = 5). Zebrafish fed diets including 0, 25, 50, 75 and 100 % of BSF prepupae meal respect to FM (Hi0, Hi25, Hi50, Hi75 and Hi100, respectively).

differences between them). Finally, as regards *lepa* gene expression (Fig. 7k), only Hi50 group was characterized by a significant downregulation with respect to Hi0. Furthermore, Hi100 group showed a significantly higher gene expression with respect to the other experimental groups fed on BSF-based diets.

Immune response. Considering genes involved in the immune response (Fig. 7l-n), groups fed the highest BSF prepupae meal inclusion levels respect to FM (Hi75 and Hi100) showed a significant upregulation with respect to Hi0, Hi25 and Hi50 groups, while no significant differences were detected among them (with the exception of Hi50 group *il10* gene expression which showed a significant upregulation than Hi0).

Chitinases. Considering genes involved in the enzymatic hydrolysis of chitin (*chia.2* and *chia.3*), Hi100 group showed the highest gene expression with respect to all the other experimental groups which did not show significant differences among them (Fig. 7o,p).

Behaviour

Open-field test. Results of the open-field test are reported in Fig. 8a,b. Diets did not show a significant effect on fish behaviour either considering the activity, measured as distance moved (ANOVA, $F_{4,75} = 0.532$, $p = 0.713$), or as the time spent in the centre of the arena ($F_{4,75} = 0.689$, $p = 0.602$). For both variables, the ANOVA test found a significant effect of time (activity: $F_{1,1119} = 446.499$; $p < 0.001$; time spent in the centre: $F_{1,1119} = 12.569$; $p < 0.001$), indicating that fish changed their behaviour over the time and therefore responded as expected to the open-field test.

Photic entrainment test. Because of the absence of a diet effect in the open field test, the behavioural photic entrainment was investigated only in two groups: Hi0 and Hi100 (Fig. 8c). The ANOVA test evidenced that fish locomotor activity significantly varied between light and dark phase with the typical pattern of diurnal species ($F_{1,5590} = 5˙590.212$, $p < 0.001$). However, the Hi0 and Hi100 fish did not show significant behavioural alterations either considering the entire testing time ($F_{1,6} = 0.008$, $p = 0.931$) or the light and dark phases ($F_{1,5590} = 0.275$, $p = 0.560$).

Discussion

In light of a sustainable circular economy, insects represent a very promising example of bio-converting organisms [**7**,**9**,**19**]. The substitution of dietary FM with BSF prepupae meal in aquafeeds is still controversial possibly due to differences in the diet formulation, the nutritional quality of insects' biomass and the fish species used during the trials [**7**,**10**,**11**].

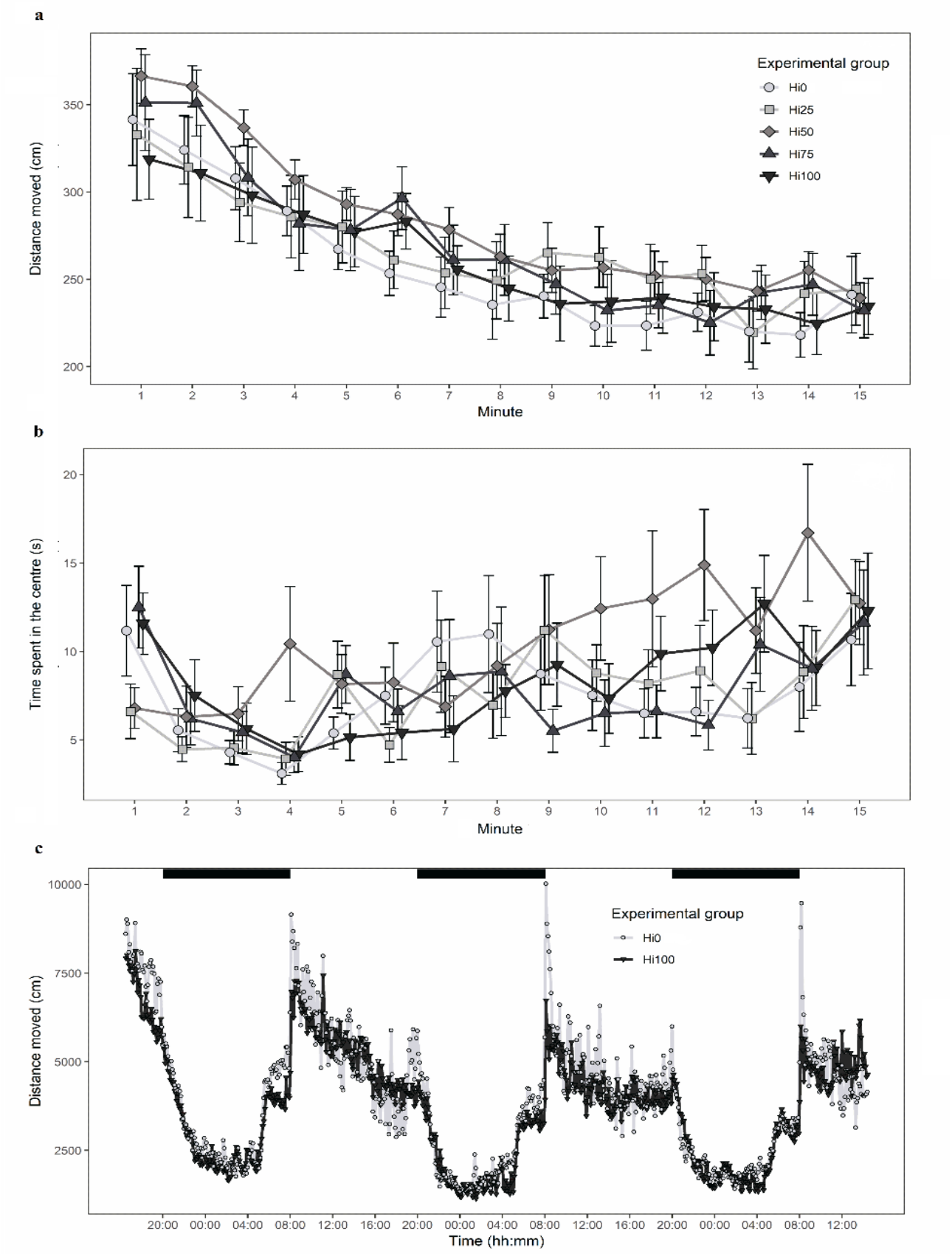


Figure 8. Behaviour**.** (**a**) activity and (**b**) time spent in the centre of the arena of zebrafish and tested in the open-field test. Dots represent means and error bars represent standard errors. Data are plotted in 1-min blocks. (**c**) Photic entrainment test: locomotor activity of zebrafish exposed to a 12 h:12 h light:dark cycle in the photic entrainment test. Black bars at the top of the graph indicate dark phases the LD cycles (light-on: 08:00, lights-off: 20:00). Dots represent means of 6-min time blocks. Zebrafish fed diets including 0, 25, 50, 75 and 100 % of BSF prepupae meal respect to FM (Hi0, Hi25, Hi50, Hi75 and Hi100, respectively).

Growth substrate selection plays a key role on the nutritional quality of BSF [**47**] and several studies have already demonstrated that the biomass of prepupae can be improved by using an organic substrate containing proper amounts of PUFA [**6**]. In particular, Truzzi et al. [**9**] demonstrated that the addition of a 10% (w/w) of *Schizochytrium* sp. to the growth substrate was an efficient method to enrich BSF prepupae final biomass in terms of PUFA and to increase, with respect to previous studies on zebrafish, the BSF prepupae meal inclusion level in the fish diets without affecting fish welfare [**21**,**29**,**48**]. However, the potential of these enriched BSF prepupae as alternative feed ingredient for fish was evaluated only during the fish larval stage [**19**].

In accord to previous studies [**11**,**12**,**49**], the present results evidenced that fish growth was positively affected by the increasing inclusion levels of enriched BSF prepupae meal respect to FM in the diets. Surprisingly, and differently from previous studies [**21**,**29**,**48**], in the present research, biometric results were not fully supported by the molecular ones that evidenced a decreasing *igf1* and *igf2a* gene expression and an opposite trend for *mstnb* in the liver, related to the increasing dietary BSF prepupae meal inclusion levels respect to FM. It should be pointed out that the principal environmental regulator of the growth hormone (GH)-IGF axis is the organisms' nutritional status [**50**]. Specifically, it is well established, that this axis can be differentially regulated by the dietary lipid content, at least at transcription level [**51**,**52**]. At this regard, Bertucci and collaborators [**50**] reported that a large dietary replacement of highly unsaturated fatty acids by saturated and monounsaturated ones caused a similar GH-IGFs pattern to that observed in starved fish. During fasting, both plasma Igfs concentration and liver *igfs* mRNA levels typically decline [**53**], while GH plasma levels raise to limit growth and stimulate lipolysis [**50**]. This condition agrees with the higher intestine *ghrl* and *cnr1* gene expression observed in Hi75 and Hi100 zebrafish groups since the regulation of orexigenic signals (ghrelin in particular) is associated with different factors including GH and Igf1 concentrations [**54**]. Furthermore, as reported by previous studies on zebrafish, fish fed BSF-based diets showed better growth performances with respect to a control diet during the larval phase [**19**,**48**], while this was not evident over a six-months feeding trial [**21**]. In the present study, a reduction in the differences of SGR% among the experimental groups was evident, especially if compared to the larval stage [**19**] (chapter 3.1), and this can be related to the well-known different growth rates of larval, juvenile and adult fish [**55**].

BSF-based diets used in this study showed a higher SFA and a lower PUFA content with respect to Hi0 diet and their lipid profile affected zebrafish fatty acids profile. However, differences among fatty acids classes were less evident in fish compared to those detected in diets because zebrafish, as a freshwater species, are able to synthesize highly unsaturated fatty acids (HUFA) starting form shorter-chain precursors through the hepatic pathways of elongation and desaturation [**56**]. In fact, in the

present study, Hi75 and Hi100 groups showed higher liver *elovl5* gene expression, a transcript that codifies for the Elovl5 enzyme that is involved in the first step of HUFA biosynthesis, compared to the other experimental groups [**56**]. Conversely, both *elovl2* and *fads2* did not show significant differences among experimental groups. However, increasing inclusion levels of BSF prepupae meal in the diets are known to affect lipid accumulation in fish liver [**13**,**21**]. In the present study, both histological and spectroscopic analyses detected a severe degree of hepatic steatosis in Hi75 and Hi100 zebrafish. This pathological condition (that caused an increase of stress markers gene expression) [**57**] has already been related to a high SFA content and a high n6/n3 ratio in the diet [**21**]. Generally, fish require proper amounts of dietary PUFA as they play an important role in the correct development of neural system [**56**]. Furthermore, altered dietary SFA and n6 intake have been related to behavioural and cognitive impairments in humans and rodents [**26**]. The FTIR analysis of brain samples evidenced that increasing inclusion levels of dietary BSF prepupae meal caused a drastic decrease of unsaturated fatty acids and carbohydrates in brain, mainly in Hi75 and Hi100 groups. Consequently, based on studies in rodents, we expected alterations of the fish behaviour [**58**,**59**]. Conversely, no dietary effects in the behavioural tests were observed suggesting that the different fatty acids composition of brain tissues was not affecting fish behaviour. Further studies are necessary to address the link between fatty acids composition in the diet and fish behaviour.

Considering intestine health status, no signs of inflammation or histopathological alterations were detected by histological analyses in the intestine samples. These results are possibly related to: (i) the digestion of chitin, a molecule that can affect diet digestibility and can alter the intestine mucosa, through the activation of specific intestinal chitinases (*chia.2* and *chia.3* which were highly expressed in Hi100 group); (ii) the anti-inflammatory, antibacterial and antiviral properties of medium-chain fatty acids (especially lauric acid, 12:0) which are particularly abundant in the BSF-based diets [**19**]; (iii) the immunomodulatory role of ghrelin. Ghrelin (highly expressed in the intestine of Hi75 and Hi100 groups) plays an important role in inflammatory responses, mainly through the regulation of cytokine production [**60**] as confirmed by the molecular markers involved in the immune response analysed in intestine samples. Specifically, *il1b*, *il10* and *tnfa* analysed in intestine samples were significantly upregulated in Hi75 and Hi100 groups, compared to the other experimental groups.

Finally, high gut microbiota biodiversity is a desirable feature because it is usually associated with a healthy host [**17**]. However, it is well established that prolonged SFA and n6 consumption is able to reduce gut microbiota variability [**27**]. Specifically, Peng et al. [**61**] reported a significant change in the composition of the intestinal microbial community of rice field eel (*Monopterus albus*) dependent on dietary lipids. In this study, dietary lipids were able to modulate the microbial diversity and, in some cases, to stimulate massive proliferation of *Cetobacterium*, suggesting that these molecules

could disturb the balance of intestinal microbiota. Similarly, in the present study, increasing inclusion levels of BSF prepupae meal respect to FM in the diets resulted in a reduction in microbiota biodiversity. This result is in contrast with previous studies reporting an increase in gut microbial richness and diversity with dietary administration of BSF meal [**16**]. However, in the present study, relative abundances of *Cetobacterium* increased in accordance with increasing dietary BSF prepupae meal inclusion levels respect to FM. A possible compensatory increasing presence of *Cetobacterium* in the gut of zebrafish fed the different experimental diets can be supported by the ability of this microbial genus to synthesize cobalamin (vitamin B12), even without a dietary source, in light to maintain cognitive capacities despite a reduction in dietary PUFA, since vitamin B12 deficiency has been related to cognitive decline [**62**,**63**].

In conclusion, while fish behaviour was not affected by the dietary treatments. results evidenced that Hi50 diet represented the best compromise between ingredient sustainability and proper fish growth and welfare. Fish fed with higher BSF prepupae meal inclusions respect to FM (75 and 100 %) showed hepatic steatosis, microbiota modification, higher lipid content, fatty acids modification and higher expression of immune response markers. Novel feed formulations based on alternative ingredients (like those here presented) are ecologically sustainable and, up to 50% of inclusion respect to FM, do not alter fish welfare, sustaining EU aquaculture priorities like productivity, sustainability and animal welfare. Over the last years the circular economy concept has become extremely important with EC Directive No. 2008/98 establishing the order of priority in the choice of waste management, the first being their reuse and the last being their landfill disposal. Insects represent excellent bio-converting organisms, which are able to convert land produced organic by-products in a valuable biological mass rich in proteins and lipids to be used in aquaculture. In this sense, the present results can sustain the aquaculture production by providing information on how to formulate sustainable practical diets in which unsustainable ingredients (FM) are substituted with sustainable ones (BSF prepupae meal). By testing different dietary BSF prepupae meal inclusion levels respect to FM, the highest inclusion level which did not affect fish growth, quality and welfare was identified (50%). Projecting these results at a global scale, a 50% inclusion of BSF prepupae meal respect to FM represents an important goal for a more sustainable aquaculture industry.

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3.3

Effects of Black Soldier Fly (*Hermetia illucens*) enriched with *Schizochytrium* sp. on zebrafish (*Danio rerio*) reproductive performances

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Abstract

The quality of nutrients in diets for broodstock is one of the major factors influencing fish oocytes maturation, reproductive success and offspring quality/survival. The Black Soldier Fly (*Hermetia illucens*; BSF) is a valuable insect species characterized by a balanced essential amino acid profile comparable to fish meal and has been demonstrated to be an eco-friendly and low-cost protein source in fish diets. However, as regards lipids, BFS is usually characterized by a poor content in polyunsaturated fatty acids which are essential for fish during critical life-cycle stages including oocyte maturation and reproduction.

In the present study, full-fat BSF prepupae meal was obtained from BSF larvae reared on coffee by-product (coffee silverskin) enriched with 10% *Schizochytrium* sp. Successively, five experimental diets containing increasing BSF prepupae meal inclusion levels respect to fish meal (0, 25, 50, 75 and 100 %) were tested during a six-month feeding trial performed on zebrafish (*Danio rerio*) to investigate growth, stress response, lipid metabolism and reproductive performances in adult females. A multidisciplinary approach including biometric, gas-chromatographic, histological and molecular analyses was used to obtain a comprehensive overview of fish responses to the test diets. Results highlighted that up to 50% of fish meal replacement with enriched BSF prepupae meal did not impair female zebrafish physiological responses while higher replacement levels (75 and 100 %) negatively affected fish stress response, oocytes maturation stages, spawning and hatching success.

Introduction

Optimal nutrition and reproductive success are two crucial aspects for achieving sustainable aquaculture development and the ability to manage fish sexual maturation and reproductive performances are essential to ensure high-quality progeny [**1**,**2**]. Therefore, a proper aquafeed composition is fundamental to broodstock nourishment [**3**,**4**] since there is evidence that nutrient quality can affect the reproductive success (with emphasis on oocyte quality) [**5**,**6**]. Teleost fish utilize the metabolic energy derived by both dietary proteins and lipids to sustain the reproduction costs [**7**,**8**], and in particular, dietary lipid and fatty acids composition of broodstock diets are recognized as the major dietary factors influencing the reproductive success and the offspring quality/survival [**9-12**]. Dietary fatty acids, particularly the unsaturated ones, play a fundamental role in gonad development, gametogenesis, spawning rate as well as embryonic and larval growth and survival [**13-15**]. Specifically, in mature female fish, polyunsaturated fatty acids (PUFA) are drained from storage sites (mainly muscle and liver) to the ovary where they are incorporated into the eggs as the main nutritional source for the embryo [**16-18**] promoting higher fertility [**19**]. Among PUFA, docosahexaenoic acid (DHA, 22:6n3) and eicosapentaenoic acid (EPA, 20:5n3) play a pivotal role

since they are involved in follicle maturation, embryo development, hatching and early larval development [**20**].

The current study was performed on the experimental model zebrafish (*Danio rerio*) because of its short life cycle, high reproductive rate and abundant information on its genomic features [**21-23**]. Results here obtained are essential to better understand the link between broodstock nutrition and female reproductive success, generalizing how several biological processes take place in model organisms providing useful information for finfish production [**21**, **24-26**]. The well-consolidated knowledge about zebrafish oocyte development and maturation [**27**,**28**] is essential to better understand the dietary effects of new diets. In this regard, it is well established that zebrafish possesses an asynchronous ovary in which different stages are easily identified: the oogonia stage (premeiotic oocyte progenitor cells) followed by the primary oocyte divided in stage IA and IB (increased in size and surrounded by the follicle layers); the stage II characterized by initial lipid deposition; a stage III, divided into stage IIIA and IIIB, during which the vitellogenesis process begins; stage IV, dominated by the fusion of yolk vesicles, and stage V characterized by mature oocytes ready for ovulation [**28**,**29**]. Finally, after the ovulation, post-ovulatory follicles (POF) composed of follicular cells are detected in the ovary [**29**]. Since different stressors are present in aquaculture conditions [**30**], including malnutrition [**31**], cultured fish often respond to these unfavourable situations by decreasing the number of mature oocytes or by increasing the number of atretic oocytes which develop through a process known as follicular atresia consisting of the degeneration of vitellogenic oocytes that fail to complete the maturation and consequently the ovulation [**32**,**33**].

Since several studies have recently been addressed in the search for new and sustainable aquafeed ingredients, deeper knowledge about the correlation between nutrition and fish reproduction success should be a sector priority. In this regard, during the last years, insects gained great attention as an alternative ingredient in aquafeed formulation thanks to their eco-friendly and cost-effective rearing and their nutritional values similar to those of fish meal (FM) [**34**]. In particular, the Black Soldier Fly (*Hermetia illucens*; BSF) is characterized by a balanced essential amino acids profile comparable to FM and represents a valuable protein source in fish diets [**35**]. However, the BSF fatty acids profile, rich in SFA and poor in PUFA is not always ideal for fish, especially when considering fish lipid requirements during the critical life-cycle stages of oocyte maturation and reproduction [**36**,**37**]. In this regard, Randazzo and collaborators [**6**] demonstrated that replacing 50% of FM with full-fat BSF prepupae meal in zebrafish females diet reduced the number of spawned eggs unbalancing their fatty acids composition (higher in SFA and lower in PUFA content) due to the typical PUFA deficiency of BSF-based diets.

However, the BSF nutritional content is deeply influenced by the rearing substrate and their fatty acids profile can be easily improved if reared on feeding substrates with a desirable amount of PUFA [**38-40**]. Previous studies used microalgae, especially marine ones, as an enrichment additive of BSF rearing substrates since they are characterized by high contents of omega-3 and omega-6 PUFA [**41**]. Zarantoniello et al. [**42**,**43**], for example, demonstrated that it was possible to replace up to 50% of FM with full-fat BSF prepupae meal in both zebrafish larvae and juveniles without impairing their growth and welfare using BSF larvae reared on a substrate enriched with 10% *Schizochytrium* sp.
In the present study, full-fat BSF prepupae meal was obtained from BSF larvae reared on coffee by-product (coffee silverskin) enriched with 10% of *Schizochytrium* sp. following the same "enrichment" procedure described in Zarantoniello et al. [**43**] (chapter 3.1). Successively, five experimental diets containing different full-fat enriched BSF prepupae meal inclusion levels (corresponding to different FM substitution levels) were tested during a six-month feeding trial to investigate the physiological effects on female zebrafish growth, stress response, lipid metabolism and reproductive performances through a multidisciplinary approach including biometric, gas-chromatographic, histological and molecular analyses.

Methods

Ethics

All the experiments involving animals were conducted in compliance with the Italian legislation on the use of animals for scientific purpose after the approval by the Ethics Committee of the Università Politecnica delle Marche (Ancona, Italy) and the Italian Ministry of Health (626/2018-PR).

Insect rearing

The feeding substrate for BSF larvae was prepared using a coffee industry by-product (coffee silverskin; moisture 44%) as the main component according to Zarantoniello et al. [**43**] (chapter 3.1) and summarized as follows. Firstly, coffee silverskin (Saccaria Caffe` S.R.L., Marina di Montemarciano, Ancona, Italy) was grounded to 2 ± 0.4 mm particle size and then 10% (w/w) of *Schizochytrium* sp. (provided freeze-dried by AlghItaly Società Agricola S.R.L., Sommacampagna, Verona, Italy) was added. A final moisture of ~70% was reached by adding distilled water to the feeding substrate. Six-day-old BSF larvae (provided by Smart Bugs s.s. Ponzano Veneto, Treviso, Italy) were individually counted and divided into groups of 640 specimens per replicate (n = 65 for a total of 41˙600 BSF larvae) and maintained following the rearing conditions reported by Zarantoniello et al. [**43**] (chapter 3.1). The feeding rate per larva was calculated at 100 mg/day according to Diener et al. [**35**] and maintained constant during the larval growth by adding new feeding substrate once a

week (448 g for each box) after the removal of the old rearing substrate. Once the prepupal stage, identified by the change in the tegument colour from white to black [**44**], was reached, insects were collected, washed, dried and stored at -80 °C.

Fish diet production

Five experimental diets were prepared using ground freeze-dried full-fat BSF prepupae using a Retsch Centrifugal Grinding Mill ZM 1000 (Retsch GmbH, Haan, Germany). The experimental diets were formulated to be grossly isonitrogenous (50% of crude proteins, N × 6.25, on dry matter) and isolipidic (13% dry matter) as previously described in Zarantoniello et al. [**12**] (chapter 3.4). A control diet (Hi0) containing FM, a vegetable protein mixture (wheat gluten and pea protein concentrate) and FO as major ingredients, was prepared according to a commercially available standard diet for zebrafish (Zebrafeed, Sparos ltd, Olhão, Portugal). The BSF-based diets were prepared by including graded levels of full-fat BSF prepupae meal to replace FM from the Hi0 formulation (approximatively 25, 50, 75 and 100 % of FM substitution, named Hi25, Hi50, Hi75, and Hi100, respectively).

Table 1. Ingredients (g/kg) and proximate composition (g/100 g) of the experimental diets used in the present study.

	Hi0	**Hi25**	**Hi50**	**Hi75**	**Hi100**
Ingredients (g/kg)					
Fish meal [1]	470	400	250	110	-
Vegetable mix [2]	220	230	298	385	440
BSF prepupae meal	-	115	235	350	460
Wheat flour [3]	198	172	120	110	72
Fish oil	80	51	25	10	-
Soy lecithin	8	8	8	11	4
Mineral and Vitamin supplements [4]	14	14	14	14	14
Binder	10	10	10	10	10
Proximate composition (%)					
Dry Matter	97.1 ± 0.1	95.8 ± 0.1	94.9 ± 0.1	93.6 ± 0.1	92.7 ± 0.1
Crude protein	51.6 ± 0.1	50.7 ± 2.6	50.4 ± 0.3	51.2 ± 1.5	50.5 ± 3.1
Ether extract	14.4 ± 0.6	13.1 ± 0.4	12.9 ± 0.4	13.2 ± 0.5	13.0 ± 0.5
Nitrogen-free extract	21.3 ± 0.3	20.8 ± 1.0	20.6 ± 0.5	19.0 ± 0.7	18.5 ± 1.3
Ash	9.8 ± 0.2	11.1± 0.01	11.0 ± 0.0	10.1 ± 0.1	10.7 ± 0.1

[1] Raw ingredient kindly supplied by Skretting Italia. [2] Vegetable mix (pea protein concentrate: wheat gluten, 0.7:1 w/w) Lombarda trading srl, Casalbuttano & Uniti (Cremona, Italy) and Sacchetto spa (Torino, Italy). [3] Consorzio Agrario (Pordenone, Italy). [4] Mineral and Vitamin supplement composition (% mix): $CaHPO_4.2H_2O$, 78.9; MgO, 2.725; KCl, 0.005; NaCl, 17.65; $FeCO_3$, 0.335; $ZnSO_4.H_2O$, 0.197; $MnSO_4.H_2O$, 0.094; $CuSO_4.5H_2O$, 0.027; Na_2SeO_3, 0.067; thiamine hydrochloride (vitamin B1), 0.16; riboflavin (vitamin B2), 0.39; pyridoxine hydrocloride (vitamin B6), 0.21; cyanocobalamine (vitamin B12), 0.21; niacin (vitamin PP or B3), 2.12; calcium pantotenate, 0.63; folic acid, 0.10; biotin (vitamin H), 1.05; myo-inositol (vitamin B7), 3.15; stay C Roche (vitamin C), 4.51; tocopherol (vitamin E), 3.15; menadione (vitamin K3), 0.24; retinol (vitamin A 2500 UI/ kg diet), 0.026; cholecalciferol (vitamin D3 2400 UI/kg diet), 0.05; choline chloride, 83.99. For proximate composition, values are reported as mean of triplicate analyses.

All the ground ingredients (0.5 mm) and FO were thoroughly blended (Kenwood kMix KMX53 stand Mixer; Kenwood, De Longhi S.p.a., Treviso, Italy) for 20 min adding water to obtain an adequate consistency for pelleting. Pellets were prepared using a 1-mm-die meat grinder, dried at 40 °C for 48-72 hours, then ground and stored in under vacuum bags at -20 °C until use. The ingredients and the proximate composition of the experimental diets are shown in Table 1.

Fish

Zebrafish embryos (wild-type line AB) were kept in a Tecniplast system (Varese, Italy) for 48 h at constant water conditions: 28 °C temperature, pH 7.0, NH_3 and NO_2^- concentrations < 0.01 mg/L, NO_3^- concentration < 10 mg/L, and a 12L/12D photoperiod. After 48 h, embryos were collected, counted under a stereomicroscope (Leica Wild M3B, Leica Microsystems, Nussloch, Germany) and randomly divided into five experimental groups (3 tanks pr experimental group) according to the five test diets.

Experimental design

Zebrafish larvae were initially reared in fifteen 20 L tanks (3 tanks per experimental group with 500 larvae per tank, 1500 larvae per dietary group). The water in the larval tanks was maintained at the same chemical-physical characteristics of the broodstock tank and was replaced 10 times a day by a dripping system [**45**]. After 30 days post fertilization (dpf), fish from each tank were gently transferred in bigger tanks (15 tanks of 80 L, 3 tanks per dietary group) equipped with mechanical and biological filtration (Panaque, Capranica, Italy). Starting from 5 dpf to 6 months, zebrafish were fed the experimental diets as follows: Hi0 group - zebrafish fed diet containing 0% of full-fat BSF prepupae meal; Hi25, Hi50, Hi75 and Hi100 groups - zebrafish fed diets including 25, 50, 75 and 100 % of full-fat BSF prepupae meal respect to FM, respectively. The feed particle sizes were adapted according to fish growth during the whole experimental period. Pellets were < 100 µm from 5 to 15 dpf, 101-200 µm from 16 to 30 dpf, 201-400 µm from 31 to 60 dpf, and 401-600 µm from 61 dpf until the end of the experiment. Fish were fed the experimental diets (2% body weight) twice a day and, from 5 to 10 dpf, rotifers *Brachionus plicatilis* (5 individual/mL) were provided to all groups, according to [**46**]. Uneaten feed and dead specimens, if present, were siphoned 30 min after feeding from all the experimental tanks. After 6 months, fish were spawned (see details in further section) and successively, part of the females was collected and anesthetized with a lethal dose of MS222 (1 g/L, Merck KGaA, Darmstadt, Germany). Liver, ovary and carcass (whole fish deprived of viscera) of female zebrafish were sampled and stored for further analyses.

Growth and reproductive parameters

At the end of the experiment (6 months), 10 female zebrafish per tank (30 females per dietary group) were randomly collected) and individually measured and weighed. Standard length and wet weight were determined by a sliding calliper (precision: 0.1 mm) and an analytical balance (precision: 0.1 mg), respectively. Additionally, livers and ovaries were individually weighted to calculate hepatosomatic (HSI) and gonadosomatic (GSI) indexes applying the following formula: [(liver weight/total zebrafish weight) × 100] for HSI and [(ovary weight/total zebrafish weight) × 100] for GSI. The survival was calculated at the end of the experiment.

Egg collection and hatching rate

According to Randazzo et al. [**6**] (chapter 2.2), 6-month-old zebrafish (7 males and 5 females, in triplicate) were randomly collected and transferred to breeding tanks (Tecniplast, Varese, Italy) (9 breeding tanks per dietary group) each day, for a period of 10 days. During the breeding period zebrafish were kept at the same water conditions of the experimental tanks (28 °C temperature, pH 7.0, NH_3 and NO_2^-concentrations < 0.01 mg/L, NO_3^- concentration < 10 mg/L); only the photoperiod was modified (14L/10D) to replicate optimal environmental conditions.

Fertilization was verified using a microscope and only eggs with a well-developed blastodisc at 3 hours post-fertilization were selected and stored at -80 °C for lipid analyses. Additionally, a proper number of fertilized eggs was used to calculate the hatching rate (150 fertilized eggs per each dietary treatment) and transferred to 10 cm-diameter Petri dishes (50 fertilized eggs per dish) containing E3 medium (5 mM NaCl, 0.17 mM KCl, 0.33 mM CaCl, 0.33 mM $MgSO_4$ to pH 7.0) and then maintained in an incubator at 28 °C until hatching (~3 dpf). Hatched larvae were finally counted, and the hatching rate was calculated for each petri dish using the formula [(hatched larvae/150) × 100].

Lipid extraction and fatty acid composition

Experimental diets, six-month-old zebrafish carcasses (whole fish deprived of the viscera; 5 females per tank, 15 females per dietary group) and just fertilized eggs (3 pools of 300 eggs per tank, 9 pools per dietary group) were analysed for lipid content and fatty acids composition. Firstly, samples were minced and homogenized (homogenizer MZ 4110, DCG Eltronic, Monza, Italy) and then freeze-dried (Edwards EF4, Crawley, Sussex, England). Aliquots of 200 mg of each sample (three aliquots per samples) were added with 100 µL of Internal Standard (methyl ester of nonadecanoic acid, 99.6%, Dr. Ehrenstorfer GmbH, Germany), and: (i) experimental diets were extracted overnight with the Folch's method [**6**]; (ii) lipid extraction in female zebrafish carcasses and just fertilized eggs were carried out on lyophilized powders following a Microwave-Assisted Extraction. All lipid extracts

were evaporated under laminar flow inert gas (N_2) until constant weight and re-suspended in 0.5 ml of n-heptane. According to Canonico et al. [**48**], fatty acids methyl esters (FAME) prepared using methyl ester of nonadecanoic acid (19:0; Dr. Ehrenstorfer GmbH) were selected as internal standard. FAME were determined by an Agilent-6890 Gas-Chromatographic (GC) System (Milano, Italy) coupled to an Agilent-5973N quadrupole Mass Selective Detector (MS) (Milano, Italy). FAME were separated using a CPS ANALITICA CC-wax-MS capillary column (30 m × 0.25 mm ID, 0.25 μm film thickness). The instrumental conditions were set up according to Truzzi et al. [**49**]. For each analysed sample, at least three runs were performed on the GC-MS.

Histology

Livers and ovaries collected at the end of the experiment from 5 female zebrafish from each tank (15 females per dietary group) were processed according to Randazzo et al. [**6**,**50**]. Samples were fixed by immersion in Bouin's solution (Merck KGaA) for 24 h at 4 °C and then washed three times (ten minutes each) with ethanol (70%) and preserved in the same ethanol solution. Samples were then subjected to a dehydration process in increasing ethanol solutions (80, 95 and 100 %), washed with xylene (Bio-Optica, Milano, Italy) and finally embedded in paraffin (Bio-Optica). Samples in solidified paraffin blocks were cut with a microtome (Leica RM2125 RTS, Nussloch, Germany) to obtain 5 μm sections that were successively stained with Mayer haematoxylin and eosin Y (Merck KGaA). Sections were observed using a Zeiss Axio Imager.A2 (Zeiss, Oberkochen, Germany) and images were acquired using a combined colour digital camera Axiocam 503 (Zeiss).

To determine the degree of fat accumulation in zebrafish females' livers, a quantitative analysis was performed on 3 sections per fish (15 female zebrafish per dietary group) collected at 50 μm intervals. The percentage of fat fraction (PFF) was calculated using the ImageJ software setting a homogeneous threshold value. Not evaluable areas such as blood vessels were excluded from the analysis.

Ovaries were fully sectioned, and a significant number of histological sections collected at 300 μm intervals from each ovary (15 ovaries per dietary group), were analysed to calculate the percentage of previtellogenic, class III, class IV, post-ovulatory follicle (POF) and atretic oocytes (based on their morphological features) on the total number of oocytes counted according to Randazzo et al. [**6**] (chapter 2.2). Oocytes were counted using the ZEN 2.3 lite Software (Zeiss).

Molecular analyses

RNA extraction and reverse transcription. Total RNA extraction from 3 pools of 2 zebrafish females' livers per tank (18 females per dietary group) was performed using RNAzol RT reagent (Merck KGaA) following the manufacturer protocol and according to Vargas-Abúndez et al. [**51**]. Final RNA

concentration was determined by the NanoPhotometer P-Class (Implen, München, Germany), while RNA integrity was verified by GelRed™ staining of 28S and 18S ribosomal RNA bands on 1% agarose gel. The reverse transcription was performed using the LunaScript RT SuperMix Kit (New England Biolabs, Ipswich, Massachusetts, USA) following the producer instructions.

Real-Time PCR. PCRs were performed according to Cardinaletti et al. [**52**] in an iQ5 iCycler thermal cycler (Bio-Rad Hercules, California, USA). The reactions were set on a 96-well plate by mixing, for each sample, 1 µL cDNA diluted 1:10, 5 µL of 2×concentrated iQ™ Sybr Green (Bio-Rad) as a fluorescent intercalating agent, 0.3 µM of both forward and reverse primers. The thermal profile for all reactions was 3 min at 95 °C followed by 45 cycles of 20s at 95 °C, 20s at the specific annealing temperature of each primer (Table 2), and 20s at 72 °C. At the end of each cycle, fluorescence was monitored, and the melting curve analyses showed in all cases the amplification specificity of the reaction. Relative quantification of the expression of genes involved in stress response (*nr3c1* and *hsp70.1*) and long-chain PUFA biosynthesis (*elovl2*, *elovl5* and *fads2*) was performed. Actin-related protein 2/3 complex, subunit 1A (*arpc1a*) and ribosomal protein large, 13 (*rpl13*) were used as endogenous standards in each sample to standardize the results by eliminating variation in mRNA and cDNA quantity and quality. Negative controls did not show amplification and primer-dimer formation was not detected in the control templates. The sequence and the homology of the amplification products was tested. Data were analysed using the iQ5 optical system software version 2.0 (Bio-Rad) which includes GeneEx Macro iQ5 Conversion and GeneEx Macro iQ5 files. Primer sequences (Table 2) were designed using Primer3 starting from zebrafish sequences available in ZFIN.

Table 2. Sequences, Zebrafish Information Network (ZFIN) IDs, and annealing temperature (A.T.) of the primers used for the present study.

Gene	Forward Primer (5'-3')	Reverse Primer (5'-3')	ZFIN IDs	A. T. (°C)
nr3c1	AGACCTTGGTCCCCTTCACT	CGCCTTTAATCATGGGAGAA	ZDB-GENE-050522-503	58
hsp70.1	TGTTCAGTTCTCTGCCGTTG	AAAGCACTGAGGGACGCTAA	ZDB-GENE-990415-91	58
elovl2	CACTGGACGAAGTTGGTGAA	GTTGAGGACACACCACCAGA	ZDB-GENE-011212-1	60
elovl5	TGGATGGGACCGAAATACAT	GTCTCCTCCACTGTGGGTGT	ZDB-GENE-040407-2	60
fads2	CATCACGCTAAACCCAACA	GGGAGGACCAATGAAGAAGA	ZDB-GENE-011212-1	60
arpc1a	CTGAACATCTCGCCCTTCTC	TAGCCGATCTGCAGACACAC	ZDB-GENE-040116-1	60
rpl13	TCTGGAGGACTGTAAGAGGTATGC	AGACGCACAATCTTGAGAGCAG	ZDB-GENE-031007-1	59

Statistical analysis

The statistical analysis was performed using the software package Prism8 (GraphPad Software) for Windows. All data were analysed by one-way ANOVA, with diet as the explanatory variable. The

ANOVA tests were followed by Tukey's post hoc test. Significance was set at $p < 0.05$ and all the results are presented as mean ± standard deviation (SD).

Results

During the whole experimental period, the fish punctually accepted all the experimental diets and the feed provided was totally consumed without rejection or loss.

Growth and reproductive parameters

As reported in Table3, Hi0 and Hi25 groups showed significantly higher standard length values compared to the Hi100 group, while Hi50 and Hi75 were characterized by intermediate ones. No significant differences were detected among the experimental groups considering wet weight, HSI and GSI.

Table 3. Biometric measurements of adult female zebrafish.

	Hi0	**Hi25**	**Hi50**	**Hi75**	**Hi100**
SL	25.6 ± 1.9 ^{a}	25.9 ± 1.7 ^{a}	25.4 ± 2.2 ^{a,b}	24.4 ± 1.9 ^{a,b}	23.8 ± 1.5 ^{b}
WW	0.35 ± 0.07	0.40 ± 0.07	0.38 ± 0.08	0.39 ± 0.12	0.36 ± 0.10
HSI	2.2 ± 0.6	2.2 ± 0.7	2.1 ± 0.5	2.9 ± 0.5	2.4 ± 0.7
GSI	11.4 ± 2.7	13.4 ± 4.4	12.7 ± 3.7	13.6 ± 2.9	12.8 ± 2.9

SL**,** Standard length (mm); WW, wet weight (g); HSI, hepatosomatic index (%); GSI, gonadosomatic index (%). Fish fed diets including 0, 25, 50, 75 and 100 % of BSF prepupae meal respect to FM (Hi0, Hi25, Hi50, Hi75 and Hi100). Different letters indicate statistically significant differences among the experimental groups ($p < 0.05$). Values are presented as mean ± SD (n = 30).

Spawned eggs and hatching rate

As reported in Figure 1a, increasing dietary inclusion levels of BSF prepupae meal replacing FM resulted in a significant decrease in the number of total spawned eggs per breeding tank over 10 days (1034 ± 29.2, 920.0 ± 17.6, 792.0 ± 31.0, 544.0 ± 23.2 and 450.7 ± 16.9 for Hi0, Hi25, Hi50, Hi75 and Hi100 respectively). Figure 1b shows the hatching rate (%). Experimental groups fed the two higher FM replacement levels (Hi75 and Hi100) with BSF prepupae meal were characterized by significantly lower hatching percentages (41.6 ± 6.6 and 47.3 ± 6.5 %, respectively) compared to the other experimental groups which did not evidence significant differences among them (76.5 ± 7.0, 74.4 ± 7.7 and 64.0 ± 9.5 % for Hi0, Hi25 and Hi50, respectively).

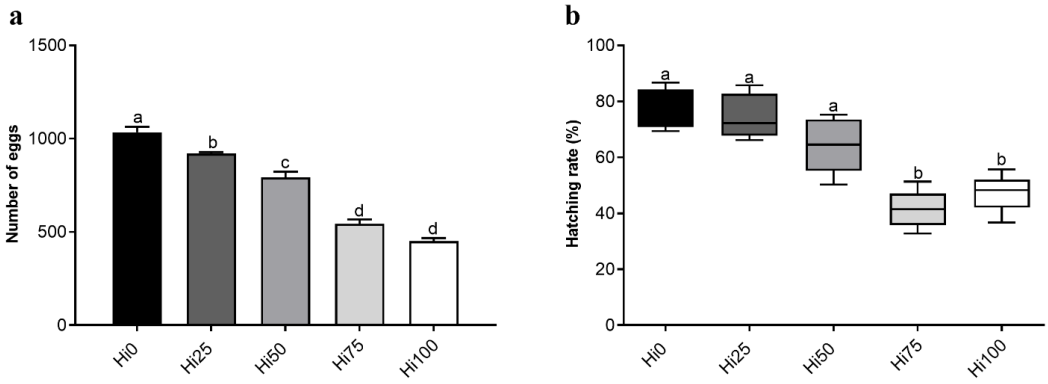

Figure 1. (**a**) Number of spawned eggs in a total period of 10 days and (**b**) hatching rate expressed in percentage, boxplots show minimum and maximum (whiskers), first quartile, median and third quartile (box). Different letters indicate significant differences among experimental groups ($p < 0.05$). Values are presented as mean ± SD (n = 10 days of spawning; n = 9 for hatching rate). Fish fed diets including 0, 25, 50, 75 and 100 % of BSF prepupae meal respect to FM (Hi0, Hi25, Hi50, Hi75 and Hi100).

Fatty acids content and composition

Diets. Results obtained from the experimental diets were previously reported in Zarantoniello et al. [**43**] (chapter 3.1). Briefly, as reported in Figure 2a, BSF-based diets showed significantly ($p < 0.05$ higher percentages of SFA and significantly lower percentages of PUFA compared to the control diet (Hi0). However, excluding the Hi0 diet, a significant increase of PUFA was evident from Hi25 to Hi100 group. Increasing dietary inclusion levels of enriched BSF prepupae meal resulted in a significant decrease of n3 percentages and a parallel significant increase in n6 percentages. Consequently, the n6/n3 ratio showed significant differences among experimental diets, increasing from Hi0 to Hi100 diet (Fig. 2b).

Female zebrafish. As reported in Figure 2c, increasing inclusion levels of BSF prepupae meal respect to FM resulted in a significant increase in SFA (24.2 ± 0.5, 27.5 ± 0.5, 30.5 ± 0.6, 31.7 ± 0.8 and 34.2 ± 1.2 % for Hi0, Hi25, Hi50, Hi75 and Hi100, respectively), monounsaturated fatty acids -MUFA- (27.6 ± 0.2, 28.7 ± 0.3, 30.4 ± 0.3, 31.0 ± 0.3 and 32.4±0.6 % for Hi0, Hi25, Hi50, Hi75 and Hi100, respectively), n6 (12.8 ± 0.2, 13.5 ± 0.2, 16.0 ± 0.2, 18.1 ± 0.3 and 20.0 ± 0.8 % for Hi0, Hi25, Hi50, Hi75 and Hi100, respectively), and n9 (17.5 ± 0.1, 18.5 ± 0.2, 20.9 ± 0.2, 22.5 ± 0.3 and 23.8 ± 0.6 % for Hi0, Hi25, Hi50, Hi75 and Hi100, respectively) percentages. Considering PUFA (48.3 ± 0.4, 43.7 ± 0.8, 39.0 ± 0.9, 37.3 ± 1.0 and 33.4 ± 1.5 % for Hi0, Hi25, Hi50, Hi75 and Hi100, respectively) and n3 (35.2 ± 0.4, 30.0 ± 0.8, 22.8 ± 0.8, 18.9 ± 1.0 and 12.9 ± 1.0 % for Hi0, Hi25, Hi50, Hi75 and Hi100, respectively) percentages, increasing inclusion levels of BSF prepupae meal resulted in a significant decrease of these fatty acid classes. Accordingly, the n6/n3 ratio (Fig. 2d) showed significant differences among experimental groups, increasing from Hi0 to Hi100 group (0.40 ± 0.04 and 1.6 ± 0.10 for Hi0 and Hi100, respectively).

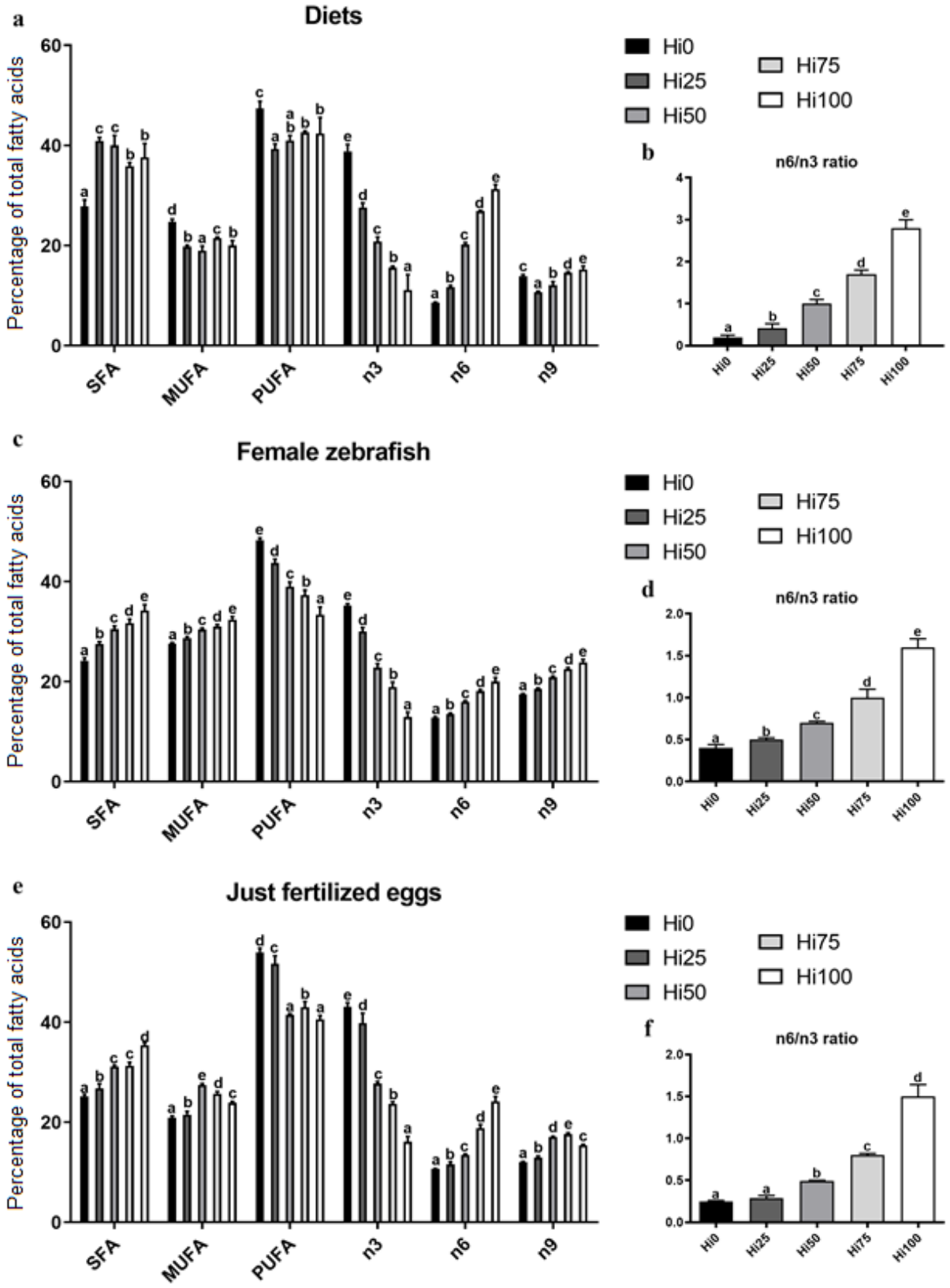

Figure 2. Content of SFA, MUFA and PUFA (as % of total fatty acids) and contribution of omega 3 (n3), omega 6 (n6) and omega 9 (n9) fatty acids to lipid profile. (**a**,**b**) experimental diets; (**c**,**d**) female zebrafish; (**e**,**f**) fertilized eggs. Fish were fed diets including 0, 25, 50, 75 and 100 % of BSF meal with respect to FM (Hi0, Hi25, Hi50, Hi75 and Hi100). Different letters indicate statistically significant differences among experimental groups compared within the same fatty acids class ($p < 0.05$). Values are presented as mean ± SD (n = 3 for experimental diets; n = 12 for female zebrafish; n = 9 for fertilized eggs).

Table 4. Fatty acids composition (% fatty acids methyl esters) of zebrafish females' carcasses and just fertilized eggs.

	Female zebrafish					Just fertilized eggs				
	Hi0	**Hi25**	**Hi50**	**Hi75**	**Hi100**	**Hi0**	**Hi25**	**Hi50**	**Hi75**	**Hi100**
10:0	0.01±0.01 ^{a}	0.04±0.01 ^{b}	0.09±0.01 ^{c}	0.09±0.01 ^{d}	0.12±0.01 ^{e}	n.d.	n.d.	n.d.	n.d.	n.d.
12:0	0.28±0.03 ^{a}	2.90±0.10 ^{b}	4.20±0.20^{c}	5.00±0.30 ^{d}	5.50±0.30 ^{e}	0.06±0.01 ^{a}	0.52±0.04 ^{b}	0.86±0.03 ^{c}	0.89±0.02 ^{c}	1.48±0.04 ^{d}
13:0	0.05±0.01 ^{a}	0.05±0.01 ^{a}	0.05±0.01 ^{a}	0.06±0.01 ^{ab}	0.07±0.01 ^{b}	0.020±0.001	0.022±0.002	0.028±0.002	0.030±0.001	0.037±0.001
14:0	4.1±0.2 ^{b}	4.2±0.1 ^{b}	4.2±0.3 ^{b}	3.7±0.3 ^{a}	4.7±0.2 ^{c}	3.1±0.2 ^{c}	2.5±0.2 ^{a}	3.3±0.1 ^{d}	2.9±0.1 ^{b}	3.6±0.1 ^{e}
14:1n5	0.58±0.10 ^{a}	0.62±0.05 ^{a}	0.69±0.12 ^{a}	0.67±0.13 ^{a}	0.88±0.12 ^{b}	n.d.	n.d.	n.d.	n.d.	n.d.
15:0	0.82±0.06 ^{c}	0.74±0.07 ^{ab}	0.69±0.06 ^{a}	0.71±0.05 ^{a}	0.80±0.06 ^{bc}	0.82±0.04 ^{b}	0.75±0.04 ^{a}	0.89±0.02 ^{c}	0.75±0.02 ^{a}	0.88±0.02 ^{c}
16:0	14.7±0.6 ^{a}	15.2±0.6 ^{a}	16.4±0.7 ^{b}	16.7±0.5 ^{bc}	17.3±0.4 ^{c}	17.4±0.3 ^{a}	17.8±0.6 ^{a}	19.0±0.4 ^{b}	19.0±0.5 ^{b}	22.8±0.3 ^{c}
16:1n9	0.75±0.07 ^{a}	0.77±0.08 ^{a}	1.30±0.10 ^{b}	1.21±0.10 ^{b}	1.75±0.20 ^{c}	1.13±0.05 ^{a}	1.30±0.07 ^{b}	1.83±0.03 ^{d}	2.32±0.07 ^{e}	1.74±0.04 ^{c}
16:1n7	6.9±0.1 ^{c}	6.9±0.1 ^{c}	6.5±0.3 ^{b}	5.8±0.1 ^{a}	5.7±0.2 ^{a}	6.0±0.3 ^{d}	5.6±0.3 ^{c}	6.3±0.1 ^{b}	5.3±0.1 ^{a}	5.3±0.1 ^{a}
16:2n7	0.25±0.01 ^{a}	0.27±0.03 ^{ab}	0.29±0.02 ^{b}	0.35±0.02 ^{c}	0.42±0.04 ^{d}	0.25±0.03 ^{a}	0.28±0.01 ^{b}	0.27±0.01 ^{ab}	0.41±0.01 ^{d}	0.36±0.01 ^{c}
17:0	0.71±0.08 ^{a}	0.80±0.07 ^{b}	0.78±0.08 ^{ab}	0.82±0.06 ^{bc}	0.90±0.09 ^{c}	0.60±0.09 ^{a}	0.89±0.03 ^{b}	0.94±0.03 ^{bc}	0.98±0.01 ^{c}	1.13±0.02 ^{d}
18:0	3.3±0.2 ^{a}	3.4±0.2 ^{a}	3.9±0.3 ^{b}	4.4±0.3 ^{c}	4.5±0.3 ^{c}	3.1±0.1 ^{a}	4.2±0.1 ^{b}	6.0±0.1 ^{c}	6.6±0.1 ^{d}	5.4±0.1 ^{e}
18:1n9	14.8±0.4 ^{a}	16.2±0.6 ^{b}	18.5±0.5 ^{c}	20.4±0.7 ^{d}	21.2±0.8 ^{e}	10.1±0.1 ^{a}	10.9±0.2 ^{b}	14.3±0.1 ^{d}	14.5±0.2 ^{e}	12.9±0.1 ^{c}
18:1n7	2.6±0.1 ^{c}	2.7±0.2 ^{c}	2.3±0.2 ^{b}	2.0±0.2 ^{a}	2.0±0.2 ^{a}	2.80±0.02 ^{a}	3.02±0.07 ^{c}	4.11±0.04 ^{e}	2.88±0.03 ^{b}	3.17±0.03 ^{d}
18:2n6	10.5±0.3 ^{a}	10.8±0.4 ^{a}	12.5±0.6 ^{b}	13.4±0.6 ^{c}	13.6±0.5 ^{c}	7.77±0.09 ^{d}	7.62±0.16 ^{c}	6.84±0.08 ^{a}	7.30±0.09 ^{b}	8.22±0.01 ^{e}
18:3n3	2.0±0.1 ^{d}	1.8±0.1 ^{cd}	1.5±0.2 ^{ab}	1.7±0.1 ^{bc}	1.4±0.3 ^{a}	1.03±0.2 ^{d}	0.87±0.03 ^{c}	0.51±0.04 ^{a}	0.48±0.01 ^{a}	0.55±0.01 ^{b}
20:0	0.16±0.01 ^{b}	0.13±0.01 ^{a}	0.12±0.01 ^{a}	0.15±0.01 ^{b}	0.15±0.01 ^{b}	0.13±0.01 ^{c}	0.12±0.01 ^{b}	0.11±0.01 ^{a}	0.10±0.01 ^{a}	0.11±0.01 ^{a}
20:1n9	1.28±0.08 ^{d}	1.03±0.07 ^{c}	0.83±0.06 ^{b}	0.70±0.06 ^{a}	0.67±0.05 ^{a}	0.77±0.01 ^{bc}	0.66±0.07 ^{a}	0.97±0.01 ^{d}	0.73±0.01 ^{b}	0.80±0.02 ^{c}
20:2n6	0.45±0.02 ^{ab}	0.42±0.03 ^{a}	0.49±0.04 ^{b}	0.66±0.05 ^{c}	0.95±0.06 ^{d}	0.60±0.01 ^{a}	0.69±0.08 ^{a}	1.00±0.01 ^{b}	1.77±0.31 ^{c}	2.42±0.12 ^{d}
20:3n6	0.54±0.03 ^{a}	0.61±0.04 ^{a}	1.01±0.05 ^{b}	1.46±0.08 ^{c}	1.77±0.10 ^{d}	1.0±0.1 ^{a}	1.2±0.1 ^{a}	2.1±0.2 ^{b}	3.0±0.4 ^{c}	4.0±0.4 ^{d}
20:4n6	1.3±0.1 ^{a}	1.6±0.1 ^{b}	1.9±0.1 ^{c}	2.5±0.2 ^{d}	3.7±0.1 ^{e}	1.27±0.05 ^{a}	2.10±0.20 ^{b}	3.50±0.20 ^{c}	6.80±0.40 ^{d}	9.50±0.80 ^{e}
20:3n3	0.21±0.02 ^{b}	0.19±0.02 ^{b}	0.15±0.03 ^{a}	0.14±0.02 ^{a}	0.15±0.03 ^{a}	0.21±0.02 ^{b}	0.23±0.01 ^{b}	0.22±0.01 ^{b}	0.17±0.02 ^{a}	0.45±0.05 ^{c}
20:5n3	13.9±0.6 ^{e}	10.8±0.5 ^{d}	6.3±0.5 ^{c}	4.3±0.3 ^{b}	2.0±0.2 ^{a}	15.70±0.20 ^{e}	11.10±0.20 ^{d}	4.00±0.10 ^{c}	2.80±0.02 ^{b}	2.19±0.05 ^{a}
22:0	0.11±0.01 ^{ab}	0.10±0.01 ^{a}	0.10±0.02 ^{a}	0.12±0.02 ^{b}	0.12±0.02 ^{b}	n.d.	n.d.	n.d.	n.d.	n.d.
22:1n9	0.75±0.05 ^{e}	0.55±0.04 ^{d}	0.30±0.04 ^{c}	0.21±0.04 ^{b}	0.11±0.02 ^{a}	n.d.	n.d.	n.d.	n.d.	n.d.
22:6n3	19.1±1.0 ^{e}	17.2±0.8 ^{d}	14.9±0.8 ^{c}	12.7±0.6 ^{b}	9.4±0.7 ^{a}	26.1±0.9 ^{d}	27.6±1.1 ^{e}	23.0±0.4 ^{c}	20.3±0.4 ^{b}	12.9±1.0 ^{a}
DHA/EPA	1.4±0.2 ^{a}	1.6±0.2 ^{a}	2.4±0.2 ^{b}	3.0±0.4 ^{c}	4.7±0.6 ^{d}	1.7±0.1 ^{a}	2.5±0.1 ^{b}	5.8±0.1 ^{c}	7.3±0.1 ^{d}	5.9±0.4 ^{c}

Different letters indicate statistically significant differences among experimental groups compared within the same matrix ($p < 0.05$). Values are presented as mean ± SD (n = 15 for female zebrafish; n = 9 for fertilized eggs). Fish fed diets including 0, 25, 50, 75 and 100 % of BSF prepupae meal respect to FM (Hi0, Hi25, Hi50, Hi75 and Hi100).

As reported in Table 4, the most represented SFA in all the experimental groups were palmitic (16:0), stearic (18:0), and myristic (14:0) acids. Considering MUFA, the predominant fatty acids in all the experimental groups were oleic (18:1n9) and palmitoleic (16:1n7) acids, whose content significantly increased and decreased, respectively, with the increasing dietary inclusion levels of BSF prepupae meal respect to FM. Linoleic (18:2n6), eicosapentaenoic (20:5n3; EPA) and docosahexaenoic (22:6n3; DHA) acids were the most abundant PUFA in all the dietary treatments. Linoleic acid and arachidonic acid (20:4n6) percentages significantly increased with the increasing dietary BSF prepupae meal content. A significant decrease in EPA percentage was detected in female zebrafish fed diets with increasing BSF meal inclusion levels, whereas as regards DHA, a similar, but milder

trend was observed compared to EPA. Consequently, the DHA/EPA ratio significantly increased with the increasing dietary BSF meal content.

Just fertilized eggs. The fatty acids content of fertilized eggs is presented in Figure 2e. The SFA (25.2 ± 0.4, 26.8 ± 0.9, 31.1 ± 0.3, 31.3 ± 0.7 and 35.4 ± 0.5 % for Hi0, Hi25, Hi50, Hi75 and Hi100, respectively) and n6 (10.6 ± 0.1, 11.6 ± 0.5, 13.5 ± 0.2, 18.8 ± 0.7 and 24.2 ± 0.9 % for Hi0, Hi25, Hi50, Hi75 and Hi100, respectively) content significantly increased with the increasing dietary inclusion levels of BSF prepupae meal, with the only exception of SFA levels that did not show a significant difference between Hi50 and Hi75 groups. MUFA concentration did not reveal a clear trend: all the experimental groups were significantly different from each other. Fish fed BSF-based diets showed significantly higher MUFA levels (21.5 ± 0.7, 27.4 ± 0.3, 25.7 ± 0.4 and 23.9 ± 0.3 % for Hi25, Hi50, Hi75 and Hi100, respectively) respect to Hi0 group (20.9 ± 0.4 %). The highest MUFA content was observed in Hi50 group followed by Hi75, Hi100 and Hi25, respectively. A similar scenario was evident for n9 contents: the highest n9 value was observed in Hi75 group (17.6 ± 0.3 %) followed by Hi50, Hi100 and Hi25 (17.0 ± 0.2, 15.4 ± 0.2 and 12.9 ± 0.3 % for Hi50, Hi100 and Hi25, respectively). Considering PUFA, Hi0 (53.9±0.8 %) revealed the significantly highest concentration followed by Hi25 (51.7±1.6 %). The PUFA levels in Hi50, Hi75 and Hi100 (41.5 ± 0.3, 43.0 ± 1.1 and 40.6 ± 0.7 % for Hi50, Hi75 and Hi100, respectively) were significantly lower compared to both Hi0 and Hi25. Finally, increasing inclusion levels of BSF prepupae meal in fish diets resulted in a significant decreasing trend of n3 content in the experimental groups (43.0 ± 0.9, 39.8 ± 2.0, 27.7 ± 0.4, 23.7 ± 0.4, 16.1 ± 1.0 % for Hi0, Hi25, Hi50, Hi75 and Hi100, respectively). These results correlated with the significant increasing values of n6/n3 ratio (Figure 2f) following the increasing BSF prepupae meal dietary levels (0.25 ± 0.01, 0.29 ± 0.03, 0.49 ± 0.01, 0.80 ± 0.02 and 1.5 ± 0.14 for Hi0, Hi25, Hi50, Hi75 and Hi100, respectively), except for Hi25 that did not show significant differences with Hi0 group.

Considering the fatty acids profile reported in Table 4, the SFA with the highest concentration in all the experimental groups were the palmitic (16:0) stearic (18:0) and myristic (14:0) acids. The palmitoleic acid (18:1n9) was the most abundant MUFA in all the experimental groups, followed by the palmitoleic acid (16:1n7). The most abundant PUFA were linoleic (18:2n6) acid, EPA (20:5n3) and DHA (22:6n3) in all the experimental groups. Considering the ARA (20:4n6) content, its concentration could be included among the most abundant PUFA if considering the Hi100 group values in both females and just fertilized eggs. EPA concentration significantly decreased in the experimental groups with the increase of BSF prepupae meal dietary inclusion levels. The same significative trend was observed for DHA contents except for Hi25 eggs that showed a significantly higher DHA level among all the other experimental groups.

Histological analysis

Liver. Increasing dietary FM substitution levels with BSF prepupae meal caused a higher fat accumulation in the liver parenchyma of female zebrafish (Fig. 3a-e) which was particularly evident in Hi75 and Hi100 dietary groups (Fig. 3d, e). The histological observations were confirmed by the PFF percentage analysis shown in figure 3f. Livers of fish fed diets including the two highest BSF prepupae meal content (Hi75 and Hi100) showed PFF values that were significantly higher compared to the other experimental groups (Hi0, Hi25 and Hi50).

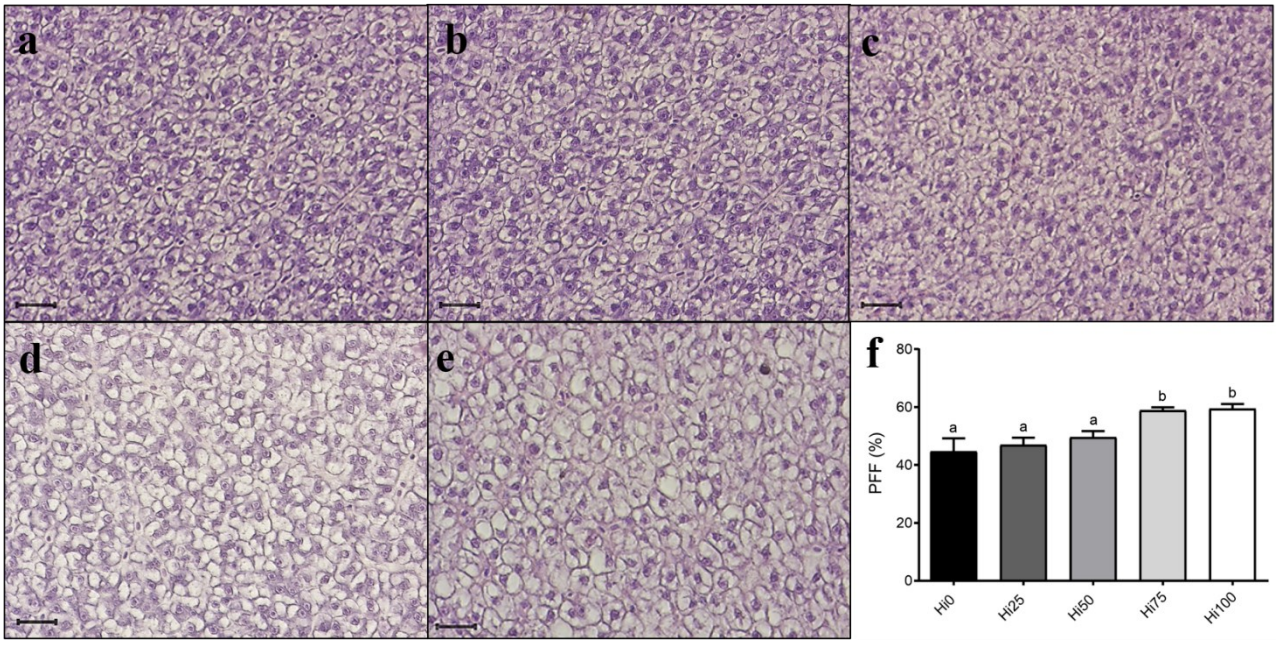

Figure 3. (**a**-**e**) Example of histomorphology of hepatic parenchyma and (**f**) percentage of fat fraction (PFF) calculate in liver samples of zebrafish females. Scale bars: 10 µm. Different letters indicate statistically significant differences among the experimental groups ($p < 0.05$). Values are presented as mean ± SD ($n = 15$). Fish fed diets including 0, 25, 50, 75 and 100 % of BSF prepupae meal respect to FM (Hi0, Hi25, Hi50, Hi75 and Hi100).

Ovary. Figure 4 shows representative histological images of zebrafish ovary. Different oocyte classes were counted [previtellogenic (PV), class III (III), class IV (IV), post-ovulatory follicles (POF) and atretic oocytes (A)] and their frequency is reported in Table 5.

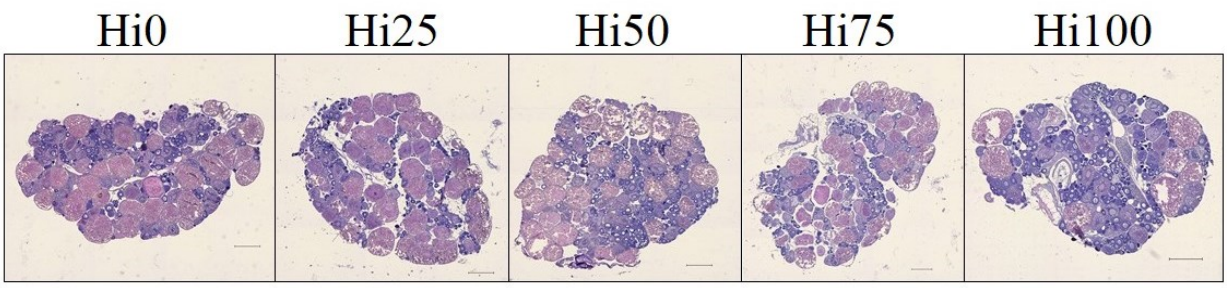

Figure 4. Histological images of zebrafish ovary histomorphology. Scale bars: 500 µm. Fish fed diets including 0, 25, 50, 75 and 100 % of BSF prepupae meal respect to FM (Hi0, Hi25, Hi50, Hi75 and Hi100).

No significant differences were detected among all the experimental groups for class IV oocytes, POF and A-stages. Conversely, the Hi100 group showed a significantly higher percentage of PV and a significantly lower percentage of class III oocytes compared to the control group (Hi0). These two stages were characterized by a slight increasing and decreasing trend (for PV and class III, respectively) in relation to BSF meal dietary inclusion level.

Table 5. Percentage of previtellogenic (PV), class III (III), class IV (IV), post-ovulatory follicles (POF), and atretic oocytes (A) detected in the present study.

	Hi0	Hi25	Hi50	Hi75	Hi100
PV	89.54 ± 4.98 ^{a}	91.58 ± 5.76 ^{ab}	91.46 ± 3.68 ^{ab}	91.46 ± 4.31 ^{ab}	97.98 ± 1.76 ^{b}
III	17.79 ± 8.11 ^{b}	9.93 ± 6.56 ^{ab}	9.21 ± 7.24 ^{ab}	8.59 ± 5.25 ^{ab}	2.02 ± 1.76 ^{a}
IV	0.41 ± 0.34	0.17 ± 0.20	0.17 ± 0.20	0.20 ± 0.15	n.d.
POF	0.06 ± 0.08	0.17 ± 0.19	0.82 ± 1.16	0.09 ± 0.08	0.04 ± 0.07
A	0.03 ± 0.05	n.d.	0.01 ± 0.01	0.08 ± 0.09	n.d.

Results are expressed as mean ± SD (n = 15). Different letters indicate statistically significant differences among the experimental groups ($p < 0.05$). Fish fed diets including 0, 25, 50, 75 and 100 % of BSF prepupae meal respect to FM (Hi0, Hi25, Hi50, Hi75 and Hi100).

Real-time PCR

Stress response. Figures 5a and b report the expression of genes involved in the stress response that was significantly upregulated in female zebrafish fed the highest BSF prepupae meal inclusion level respect to FM (Hi100) compared to the other experimental groups (except for the *nr3c1* gene expression in Hi75 group). No significant differences were detected among the other experimental groups.

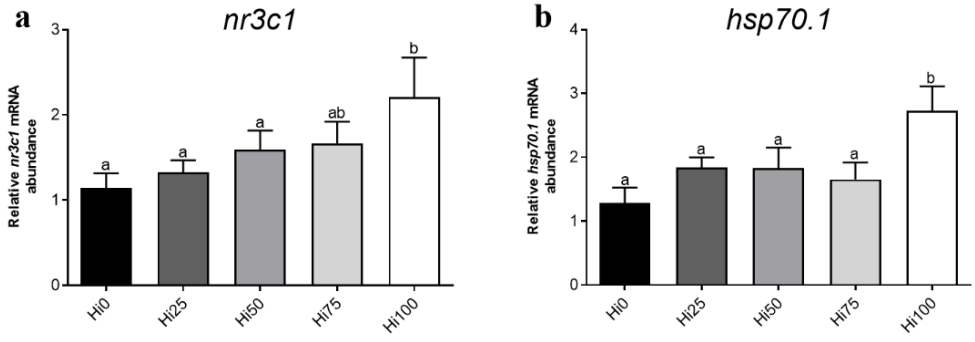

Figure 5. Relative mRNA levels of genes involved in stress response analysed in liver samples from adult female zebrafish. (**a**) *nr3c1* and (**b**) *hsp70.1*. Different letters indicate statistically significant differences among experimental groups ($p < 0.05$). Values are presented as mean ± SD (n = 9). Fish fed diets including 0, 25, 50, 75 and 100 % of BSF prepupae meal respect to FM (Hi0, Hi25, Hi50, Hi75 and Hi100).

Lipid metabolism. As reported in Figures 6a and b, female zebrafish fed the higher BSF prepupae meal inclusions respect to FM (Hi75 and Hi100) showed a significant *elovl2* and *elovl5* upregulation compared to the other experimental groups (except for *elovl5* gene expression in the Hi75 group). Furthermore, the higher was the BSF meal inclusion respect to FM, the higher was the *fads2* gene expression (Fig. 6c). However, no significant differences were evident among Hi25, Hi50 and Hi75 groups and between Hi75 and Hi00 groups.

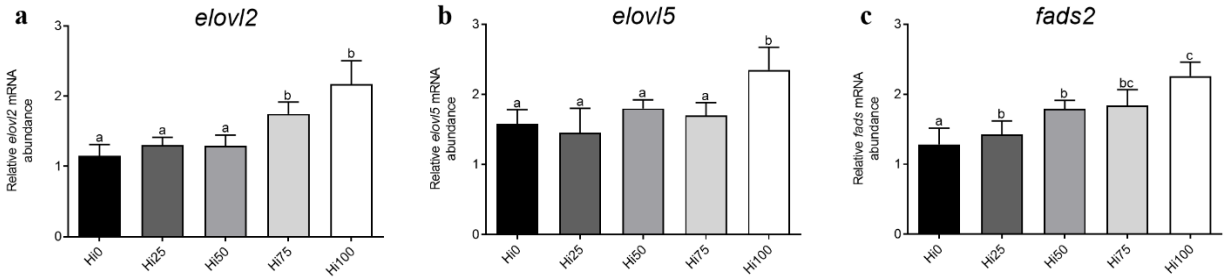

Figure 6. Relative mRNA levels of genes involved in lipid metabolism analysed in liver samples from adult female zebrafish. (**a**) *elovl2*, (**b**) *elovl5* and (**c**) *fads2*. Different letters indicate statistically significant differences among experimental groups ($p < 0.05$). Values are presented as mean ± SD ($n = 9$). Fish fed diets including 0, 25, 50, 75 and 100 % of BSF prepupae meal respect to FM (Hi0, Hi25, Hi50, Hi75 and Hi100).

Discussion

Up to now, most scientific research on the application of insect meal in aquafeed formulation has mainly focused on the effects of different dietary inclusion levels on fish physiological responses during the grow-out phase with emphasis on the nutritional edible portion quality [**53-56**]. Presently, the effects of insect-based diets on broodstock reproduction remain to be deeply investigated.

Oocyte development and reproduction are delicate processes that are known to be deeply influenced by the quality of broodstock nutrition. The essential fatty acids derived from dietary lipids influence the biochemical composition of the yolk, and consequently the embryonic development [**57**]. Long-chain PUFA like ARA and EPA are important structural components of cell membrane phospholipids [**58**] and represent the two most important eicosanoid precursors involved in follicle maturation, hatching and early larval development in fish [**59**,**60**]. Whereas an adequate supply of DHA in eggs is crucial to support neural tissue development during the embryogenic phase [**61**].

The BSF prepupae used in the present study were reared on a coffee-silverskin substrate enriched with *Schizochytrium* sp. to improve their fatty acids profile (especially DHA, EPA, and at minor extent ARA), as demonstrated by Truzzi et al. [**40**]. As previously described by Zarantoniello et al. [**42**-**43**], the "enriched" BSF prepupae meal could be included in diets for zebrafish larvae and juveniles replacing up to 100% of FM without impairing fish growth. The experimental diets used in these studies and in the present trial were formulated using a mixture of highly digestible wheat gluten

and pea protein concentrate since, to maintain a constant protein and lipid content in all the experimental diets, it was necessary to considerably increase some vegetable ingredients. However, these ingredients have already been demonstrated to not affect growth of zebrafish and other fish species [**62-65**]. According to the previous works on zebrafish larvae and juveniles [**42**,**43**], adult female zebrafish well adapted to the experimental diets throughout the entire feeding trial as the inclusion of "enriched" BSF prepupae meal did not affect neither growth performance (except for body length) nor the somatic indexes, supporting, also in the present study, the feasibility to replace FM during zebrafish culture from the early larval stage (5 dpf) to adults (6 months).

Considering the fatty acids content, both the carcasses of female zebrafish and the fertilized eggs reflected the n3 and n6 PUFA trends of the experimental diets. Indeed, it is well known that the dietary fatty acids profile is one of the most important factors that influence the fatty acids profile of fish tissues and eggs [**20**,**66**,**67**]. In particular, the increasing dietary FM substitution levels with BSF prepupae meal caused a decrease of EPA and DHA and a parallel increase of ARA content in both females' carcasses and eggs. This trend is in accord with previous studies which showed a similar fatty acids profile in freshwater species fed on diets containing limited dietary long-chain PUFA [**6**,**68-70**]. In the present study, the lower amount of EPA and DHA detected in females' carcasses and eggs of fish fed BSF-based diets was related to a reduction in the number of spawned eggs. However, this was not evident for the hatching rate, which only showed a significant reduction in Hi75 and Hi100 groups. Previously, Randazzo and collaborators [**6**] obtained similar results after testing two experimental diets where both 25 and 50 % of FM were replaced with a full-fat not-enriched BSF prepupae meal in female zebrafish. The authors related the reduction of total spawned eggs to an oocyte maturation delay due to the lack of dietary PUFA. Our results also supported this hypothesis since histological analyses on the ovary showed an increase in previtellogenic oocytes and a parallel decrease of stage III oocytes with the increasing dietary BSF prepupae meal inclusion levels. However, as already suggested by Randazzo et al. [**6**], the limited supply of dietary EPA and DHA has been probably obviated, to some extent, by the *ex-novo* synthesis of these long-chain PUFA by female zebrafish. Indeed, in comparison to marine species which lack the ability to synthesize long-chain PUFA, freshwater fish species manage to synthesize both EPA and DHA from the shorter-chain precursors (as the *α*-linolenic acid) [**37**]. In the current study, the activation of this pathway was suggested by the higher gene expression of *elovl2*, *elovl5* and *fads2* observed in fish fed BSF-based diets, especially at the two highest inclusion levels (Hi75 and Hi100), supporting the hypothesis that the primary mechanism responsible for increased PUFA biosynthesis during limited dietary long-chain PUFA intake relies in the upregulation of these genes [**66**].

Therefore, the ability of female zebrafish to produce proper quality eggs may depend on the adequate amount of EPA and DHA or of shorter-chain precursors provided by the diets. Indeed, female zebrafish fed BSF-based diets characterized by 25 and 50 % of FM replacement with enriched BSF meal were able to produce less, but good-quality eggs without compromising the hatching success. Conversely, at higher BSF dietary inclusion levels with respect to FM (Hi75 and Hi100), the significant reduction of both spawned eggs and hatching success is probably related to an inadequate dietary intake and/or biosynthesis of long-chain PUFA which led to an EPA and DHA content in the eggs probably below a threshold limit to ensure a successful hatching.

Finally, also the liver plays a pivotal role in female fish reproduction since this organ is involved in the vitellogenin synthesis before its transport to the ovary [**71**]. The present study showed a severe degree of hepatic steatosis in zebrafish fed Hi75 and Hi100 diets as indicated by the PFF values. The hepatic fat accumulation has previously been attributed to both high SFA content and high n6/n3 ratio of BSF-based diets [**52**,**72-74**]. Since hepatic steatosis is a pathological condition for fish, the significant upregulation of the stress markers here analysed well fits with the Hi100 group status that showed the most severe fat accumulation. According to these results, previous studies performed on both zebrafish and finfish species like rainbow trout showed a correlation between hepatic steatosis and overexpression of stress markers in fish fed increasing BSF prepupae meal dietary levels [**42**,**52**]. Since stress has a negative impact on fish welfare and reproduction [**30**,**75**], the *scenario* observed in the present study represents one more unfavourable physiological condition interfering with the reproductive success of Hi75 and Hi100 groups.

As a conclusion, the present study highlighted that up to a 50% FM replacement with enriched BSF meal did not impair zebrafish reproduction. Higher replacement levels (Hi75 and Hi100) negatively affected fish stress response, oocytes maturation stages, spawning and hatching success. In the light of these results, further research is necessary to identify different approaches to further improve the fatty acids quality of the insect biomass to sustain proper fish reproductive success.

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3.4

Possible dietary effects of insect-based diets across zebrafish (*Danio rerio*) generations: a multidisciplinary study on the larval phase

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Abstract

Insects represent a valuable and sustainable alternative ingredient for aquafeed formulation. However, insect-based diets have often highlighted controversial results in different fish species, especially when high inclusion levels were used. Several studies have demonstrated that nutritional programming through parental feeding may allow the production of fish better adapted to use suboptimal aquafeed ingredients. To date, this approach has never been explored on insect-based diets. In the present study, five experimental diets characterized by increasing full-fat Black Soldier Fly (*Hermetia illucens*; BSF) prepupae meal inclusion levels respect to fish meal (0, 25, 50, 75 and 100 %) were used to investigate the effects of programming via broodstock nutrition on F1 zebrafish larvae development. The responses of offspring were assayed through biometric, gas chromatographic, histological, and molecular analyses. The results evidenced that the same BSF-based diets provided to adults were able to affect F1 zebrafish larvae fatty acids composition without impairing growth performances, hepatic lipid accumulation and gut health. Groups challenged with higher BSF prepupae meal inclusions with respect to fish meal (50, 75 and 100 %) showed a significant downregulation of stress response markers and a positive modulation of inflammatory cytokines gene expression. The present study evidenced that nutritional programming through parental feeding may make possible to extend the fish meal substitution level with BSF prepupae meal in the diet up to almost 100% without incurring the well-known negative side effects of BSF-based diets.

Introduction

The partial or complete replacement of fish meal (FM) and fish oil (FO) in aquafeed formulations represents an ongoing challenge in the aquaculture sector [**1**,**2**]. For this reason, improvements in the use of low-cost and more sustainable alternative ingredients are considered a priority for the further development of fish culture [**3**]. In light of the circular economy concept and the importance of by-product reuse, insects have gained great attention as a promising aquafeed ingredient due to their nutritional value, bio-converting efficiency and their low environmental requirements [**4**,**5**]. In particular, the Black Soldier Fly (*Hermetia illucens*; BSF) is able, during its larval development, to grow on organic by-products, converting them into valuable biomass with a nutritional composition dependent on the quantity and the quality of feed offered [**6-8**]. Due to its promising protein content and essential amino acid pattern, the use of different BSF dietary inclusion levels has been widely investigated in several farmed fish species, including rainbow trout (*Oncorhynchus mykiss*) [**9**,**10**], Atlantic Salmon (*Salmo salar*) [**11**,**12**] and Siberian sturgeon (*Acipenser baerii*) [**13**,**14**], as well as in experimental models like zebrafish (*Danio rerio*) [**8**,**15**,**16**]. In addition, as recently reviewed by

Zarantoniello and collaborators [**17**], different diets in terms of BSF dietary inclusions, developmental stage (larvae or prepupae) and lipid content (full-fat, partially or totally defatted) have been tested in recent years on several fish species. However, the BSF fatty acids profile (characterized by high content of medium-chain saturated -SFA- and monounsaturated -MUFA- fatty acids, rather than long-chain polyunsaturated -PUFA- ones) [**18**] on fish growth, welfare and quality still deserve a deeper investigation, especially over a long-term period.

Only a small number of studies have been performed considering the effects of BSF-based diets on the whole life cycle of fish [**15**,**19**] and, to our knowledge, none on the progeny. It has been demonstrated that parental diet, especially in terms of n3 and n6 PUFA profile, can affect oocyte composition, embryonic development as well as progeny health in different vertebrate species [**20-22**], including teleost fish [**19**,**23**,**24**]. In this regard, there is evidence that the environmental factors experienced by parents, including nutrient availability during growth and reproduction, can have long-lasting effects on offspring metabolism [**25**,**26**]. In particular, in fish, maternally derived nutrients have direct impacts on the progeny during embryogenesis, endogenous feeding period and beyond yolk exhaustion [**27-29**]. Differently from mammals which encounter fluctuations in maternal nutrients, hormones and metabolites during gestation and lactation, the early nutritional environment of fish larvae (from fertilization to yolk absorption) is fixed before fertilization [**30**]. For that reason, parental feeding is very effective for nutritional programming in fish, showing substantial effects on progeny metabolism, growth, survival and transcriptional profile [**31-33**]. The exposure to dietary stimulus during critical fish life cycle stages can lead to long-term changes in metabolic processes, in a phenomenon called nutritional programming [**27**].

In aquaculture, nutritional programming can be useful to produce fish that are more adapted to the farming conditions and better accustomed to use alternative dietary ingredients lacking specific macronutrients [**34**,**35**]. In this regard, it has been demonstrated that partial replacement (60%) of FO with linseed oil in gilthead seabream (*Sparus aurata*) broodstock diets induced long-term persistent effects on the progeny, which showed higher ability to use low FM and FO -based diets even after 16 months post hatching [**36**,**37**]. This adaptation was dependent on the regulation of hepatic lipid metabolism which, in turn, induced positive and persistent changes in the progeny PUFA profile [**26**,**37**,**38**]. In this sense, given the importance of long-chain PUFA for human health [**39**], as well as the increasingly limited availability of aquafeed ingredients rich in these fatty acids [**40**], nutritional programming may adjust fish metabolism to maximize the ability of farmed fish to use specific ingredients, with an emphasis on dietary fatty acids [**36-38**].

The present study aimed to investigate, for the first time, whether nutritional programming exists in zebrafish larvae fed diets including increasing dietary levels of full-fat BSF prepupae meal, with emphasis on fish growth, health and fatty acids composition.

Even though zebrafish is a widely used model organism, information about its dietary predilections and nutritional requirements is mostly unknown [**41**]. The natural diet of wild zebrafish is composed of a wide variety of benthic and planktonic crustaceans, worms and insect larvae [**42**]. However, the analysis of wild zebrafish gut contents evidenced that insects, mostly of terrestrial origin, represent their main prey [**43**,**44**]. In this regard, a previous study by Vargas and collaborators [**45**] pointed out that a 100% BSF prepupae meal diet did not affect zebrafish larval development over the course of a 21-day experiment. However, in laboratory conditions, zebrafish are known to be regularly fed commercial diets (i.e., Zebrafeed, Sparos ltd, Olhão, Portugal). Since zebrafish represents an extraordinary experimental model for aquaculture studies, contributing to our understanding of how mechanisms involved in fish nutrition, welfare and growth take place in farmed fish species [**46**], testing new dietary formulations with the inclusion of insect meal is necessary for the further development of the aquaculture industry. Finally, due to its complete genome availability and relatively short life cycle, zebrafish allow us to investigate possible dietary effects, and their eventual persistence, throughout the whole life of the fish and across generations, in a relatively short time [**31**,**46**,**47**].

Methods

Insect rearing

The BSF larvae feeding substrate, consisting mainly of coffee silverskin (a coffee industry by-product), was prepared according to Zarantoniello et al. [**8**] (chapter 3.1). Briefly, before the feeding substrate preparation, coffee by-product (Saccaria Caffé S.R.L., Marina di Montemarciano, Ancona, Italy; moisture 44%) was ground in an Ariete 1769 food processor (De'Longhi Appliances Srl, Treviso, Italy) to a particle size of 2 ± 0.4 mm. The feeding substrate was formulated including a 10% (w/w) of *Schizochytrium* sp. (provided freeze-dried by AlghItaly Società Agricola S.R.L., Sommacampagna, Verona, Italy) to the coffee by-product. To reach a final moisture of ~70% in the feeding substrate [**48**], distilled water was added. Six-day-old BSF larvae (Smart Bugs s.s. Ponzano Veneto, Treviso, Italy) were divided into groups of 640 specimens per replicate (n = 65 for a total of 41˙600 BSF larvae). Each replicate consisted of a plastic box (57 × 38 × 16 cm) screened with fine-mesh cotton gauze and covered with a lid with 90 ventilation holes of 0.05 cm diameter [**49**]. BSF larvae were reared in a climatic chamber at a temperature of 27 ± 1 °C and relative humidity of 65 ± 5 % [**49**], at a density of 0.3/cm^2 [**50**], in continuous darkness. The feeding rate per larva was 100

mg/day [**51**], achievable by adding new feeding substrate once a week (448 g for each box). At the prepupal stage, identified by the change in tegument colour from white to black [**52**], insects were collected, washed, freeze-dried and stored at -80 °C.

Fish Diets

Freeze-dried full-fat BSF prepupae were ground with a Retsch Centrifugal Grinding Mill ZM 1000 (Retsch GmbH, Haan, Germany) for experimental diet preparation. Five experimental diets were prepared as previously described in Zarantoniello et al. [**8**] (chapter 3.1).

Table 1. Ingredients (as g/Kg) and proximate composition (g/100g) of the experimental diets used in the present study according to Zarantoniello et al. [**8**] (chapter 3.1).

	Hi0	**Hi25**	**Hi50**	**Hi75**	**Hi100**
Ingredients (g/kg)					
Fish meal [1]	470	400	250	110	-
Vegetable protein mix [2]	220	230	298	385	440
BSF prepupae meal	-	115	235	350	460
Wheat flour [3]	198	172	120	110	72
Fish oil	80	51	25	10	-
Soy lecithin	8	8	8	11	4
Mineral and Vitamin supplements [4]	14	14	14	14	14
Binder	10	10	10	10	10
Proximate composition (%)					
Moisture	2.9 ± 0.1	4.2 ± 0.1	5.1 ± 0.1	6.5 ± 0.1	7.3 ± 0.1
Crude protein, CP	51.6 ± 0.1	50.7 ± 2.6	50.4 ± 0.3	51.2 ± 1.5	50.5 ± 3.1
Crude lipid, CL	14.4 ± 0.6	13.1 ± 0.4	12.9 ± 0.4	13.2 ± 0.5	13.0 ± 0.5
Nitrogen-free extract	21.3 ± 0.3	20.8 ± 1.0	20.6 ± 0.5	19.0 ± 0.7	18.5 ± 1.3
Ash	9.8 ± 0.2	11.1± 0.01	11.0 ± 0.00	10.1 ± 0.1	10.7 ± 0.1
Fatty acids content (as % of total fatty acids)					
SFA	27.8±1.3 ^{a}	40.9±0.7 ^{c}	40.0±2.0 ^{c}	35.9±0.7 ^{b}	37.6±2.8 ^{b}
MUFA	24.7±0.6 ^{d}	19.8±0.3 ^{b}	19.0±0.9 ^{a}	21.5±0.2 ^{c}	20.0±1.0 ^{b}
PUFA	47.4±1.4 ^{c}	39.3±1.0 ^{a}	41.0±1.0 ^{ab}	42.6±0.3 ^{b}	42.4±3.2 ^{b}
n3	38.8±1.4 ^{e}	27.6±0.9 ^{d}	20.8±0.9 ^{c}	15.6±0.3 ^{b}	11.1±3.1 ^{a}
n6	8.6±0.1 ^{a}	11.7±0.3 ^{b}	20.2±0.4 ^{c}	26.9±0.1 ^{d}	31.3±0.9 ^{e}
n9	13.9±0.3 ^{c}	10.7±0.2 ^{a}	12.1±0.7 ^{b}	14.6±0.2 ^{d}	15.2±0.7 ^{e}
n6/n3	0.22±0.05 ^{a}	0.42±0.10 ^{b}	1.00±0.10 ^{c}	1.70±0.10 ^{d}	2.80±0.20 ^{e}

[1] Raw ingredient kindly supplied by Skretting Italia. **[2]** Vegetable protein mix (pea protein concentrate : wheat gluten, 0.6:1 w/w in all the experimental diets) provided by Lombarda trading srl (Casale Belvedere, CR, Italy) and Sacchetto spa (Lagansco, CN, Italy). **[3]** Consorzio Agrario (PN, Italy); **[4]** Mineral and Vitamin supplement composition (% mix): $CaHPO_4 2H_2O$, 78.9; MgO, 2.725; KCl, 0.005; NaCl, 17.65; $FeCO_3$, 0.335; $ZnSO_4.H_2O$, 0.197; $MnSO_4.H_2O$, 0.094; $CuSO_4.5H_2O$, 0.027; Na_2SeO_3, 0.067; thiamine hydrochloride (vitamin B1), 0.16; riboflavin (vitamin B2), 0.39; pyridoxine hydrocloride (vitamin B6), 0.21; cyanocobalamine (vitamin B12), 0.21; niacin (vitamin PP or B3), 2.12; calcium pantotenate, 0.63; folic acid, 0.10; biotin (vitamin H), 1.05; myo-inositol (vitamin B7), 3.15; stay C Roche (vitamin C), 4.51; tocopherol (vitamin E), 3.15; menadione (vitamin K3), 0.24; retinol (vitamin A 2500 UI/kg diet), 0.026; cholecalciferol (vitamin D3 2400 UI/kg diet), 0.05; choline chloride, 83.99. For proximate composition and fatty acid content, values reported as mean ± SD (n = 3). Different letters show statistically significant differences among experimental groups compared within the same FA class ($p < 0.05$)

Briefly, diets were formulated to be grossly iso-nitrogenous (50% of crude protein, N × 6.25, on dry matter) and iso-lipidic (13% on dry matter). A control diet (Hi0) containing FM, a vegetable protein mixture (wheat gluten and pea protein concentrates) and FO as major ingredients was prepared and used as the basal diet formulation for the tested BSF-based diets. BSF-based diets were prepared by including graded levels of full-fat BSF prepupae meal (approximatively 25, 50, 75 and 100 % named Hi25 and Hi50, Hi75 and Hi100, respectively) in the Hi0 formulation to replace FM. To maintain the diets iso-nitrogenous and iso-lipidic condition, the vegetable protein mixture was adjusted accordingly. In summary, all the ground ingredients (0.5 mm) were thoroughly blended (Kenwood kMix KMX53 stand Mixer; Kenwood, De Longhi S.p.a., Treviso, Italy) for 20 min, adding water to obtain an appropriate consistency for pelleting. Pellets were obtained through the use of a 1-mm-die meat grinder, dried at 40 °C for 48-72 h and then ground, sieved and stored in vacuum bags at -20 °C until use. Ingredients and proximate composition of the test diets are reported in Table 1.

Broodstock rearing and F0 production

Zebrafish AB strain adults (broodstock; 1.2 ± 0.4 g), fed on a commercial diet (Blue Line, Macerata, Italy), were laboratory spawned and zebrafish AB embryos (F0) were maintained 48 h in a Techniplast system (Varese, Italy) at 28 °C, pH 7.0, NH_3 and NO_2^- concentrations < 0.01 mg/L, NO_3^- concentration < 10 mg/L and photoperiod 12 h light/12 h dark. After this first period, embryos were gently collected, counted under a stereomicroscope and randomly assigned to the different experimental groups: F0Hi0, F0Hi25, F0Hi50, F0Hi75 and F0Hi100 (Figure 1). Fish were initially kept in 15 tanks (20 L, three tanks per dietary group with 500 fish per tank), the sides of which were covered with black panels to reduce light reflection [**53**]. The water in the F0 larval tanks had the same chemical-physical characteristics of the broodstock's tank and was gently replaced 10 times a day by a dripping system. Starting from 5 days post fertilization (dpf), fish from each dietary group were fed the same experimental diet (Hi0, Hi25, Hi50, Hi75 and Hi00 diets, respectively; 2% body weight) twice a day and, in addition, from 5 to 10 dpf, rotifer *Brachionus plicatilis* (five individuals per mL) were provided to all dietary groups (one feeding in the morning). At 21 days dpf, the required F0 larvae from each experimental group (F0Hi0, F0Hi25, F0Hi50, F0Hi75 and F0Hi00, respectively) were sampled, euthanized with a lethal dose of MS222 (1g/L; Merck KGaA, Darmstadt, Germany) and properly stored for further analyses (for details, please see Zarantoniello et al. [**8**] - chapter 3.1). Finally, at 30 dpf, the remaining F0 zebrafish (~200 fish per tank, ~600 per dietary group) were transferred, according to each experimental group, in 15 bigger tanks (80 L; 3 tanks per dietary group) equipped with mechanical and biological filtration (Panaque, RM, Italy) until 180 dpf. Feed particle sizes were <100 μm from 5 to 15 dpf, 101-200 μm from 16 to 30 dpf, 201-400 μm from 31 to 60 dpf

and 401-600 µm from 61 to 180 dpf. After 180 dpf, adult F0 zebrafish were spawned and progeny embryos (F1; Figure 1) were obtained from each dietary group.

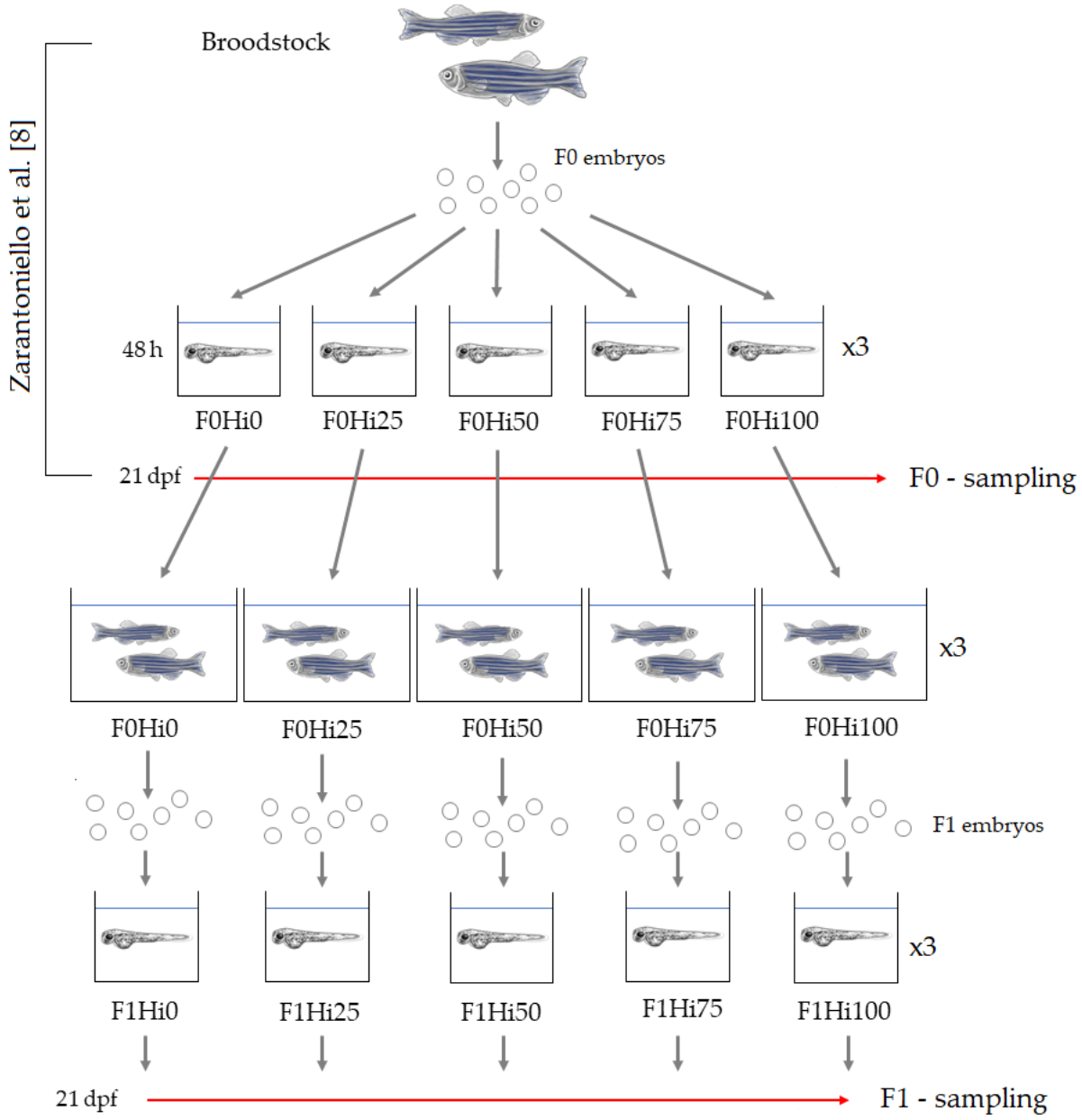


Figure 1. Schematic representation of the experimental design. Fish fed diets including 0, 25, 50, 75 and 100 % of BSF prepupae meal respect to FM (F0Hi0, F0Hi25, F0Hi50, F0Hi75 and F0Hi100 for F0 zebrafish and F1Hi0, F1Hi25, F1Hi50, FiHi75 and F1Hi100 for F1 zebrafish larvae). dpf - days post fertilization; F0 - parental generation; F1 - first filial generation.

F1 zebrafish larvae

First filial generation (F1) embryos, obtained from each experimental group (F1Hi0, F1Hi25, F1Hi50, F1Hi75 and F1Hi00, respectively) were gently collected, counted under a stereomicroscope and transferred to 20 L tanks (three tanks per dietary group). Each experimental group, in triplicate, was composed of 1500 larvae (500 larvae per tank). Starting from 5 to 20 dpf, F1 larvae were fed on the same parental diet twice a day (2% body weight; 100-200 µm particle size) and were named as: (i)

F1Hi0 (control) group: F1 larvae fed parental diet with 0% of full-fat BSF prepupae meal; (ii) F1Hi25, F1Hi50, F1Hi75 and F1Hi100 groups: F1 larvae fed parental diet including 25, 50, 75 and 100 % of full-fat BSF prepupae meal respect to FM, respectively. Furthermore, from 5 to 10 dpf, all groups were fed (one feeding in the morning) on the rotifer *Brachionus plicatilis* (5 ind/mL). All the tanks were siphoned 30 min after feeding (two times a day) to remove possible feed excess and dead larvae which were counted to estimate the survival rate. The required F1 larvae (for details, please see further sections) were sampled at 20 dpf, euthanized with a lethal dose of MS222 (1 g/L; Merck KGaA, Darmstadt, Germany) and properly stored for further analyses.

Biometry

Ten F1 zebrafish larvae (30 per dietary group) were randomly collected from each tank at hatching (3 dpf) and at 21 dpf. Excess water was removed by means of a syringe, and wet weight was determined by an analytical balance (precision: 0.1 mg) by measuring five larval pools at 3 dpf and single specimens at 21 dpf. For each experimental group, specific growth rate (SGR) was calculated as follows: SGR% = (lnWf - lnWi) / t) × 100, where Wf is the final wet weight, Wi, the initial wet weight, and t, the number of days (17). Survival rate was evaluated by subtracting the number of dead larvae at 21 dpf to the initial number (500 per tank).

Fatty acids composition

Experimental diets and F1 zebrafish larvae samples were analysed (in triplicate) for fatty acids composition according to Zarantoniello et al. [**8**] (chapter 3.1). Briefly, samples were minced and homogenized (homogenizer MZ 4110, DCG Eltronic, Monza, Italy), and larvae were also freeze-dried (Edwards EF4, Crawley, Sussex, UK). Aliquots of 200 mg of each sample (three aliquots per sample) were added with 100 µL of Internal Standard (methyl ester of nonadecanoic acid, 99.6%, Dr. Ehrenstorfer GmbH, Augsburg, Germany), and lipid extraction was carried out on lyophilized powders following a microwave-assisted extraction [**54**]. All lipid extracts were evaporated under laminar flow inert gas (N_2) until constant weight and re-suspended in 0.5 mL of n-epthane. Fatty acids methyl esters (FAME) were prepared according to Canonico et al. [**55**] and were determined using an Agilent-6890 GC System (Milano, Italy) coupled to an Agilent-5973 N quadrupole Mass Selective Detector (MSD) (Milano, Italy) and separated through a CPS ANALITICA CCwax-MS (30 m × 0.25 mm ID, 0.25 µm film thickness) capillary column [**56**]. For each analysed aliquot of sample, at least three runs were performed on the GCMS.

Histology

Five F1 zebrafish larvae (15 per dietary group) were randomly collected from each tank at 21 dpf, fixed by immersion in Bouin's solution (Merck KGaA, Darmstadt, Germany) and then stored at 4 °C for 24 h. Samples were washed three times with ethanol (70%) for 15 min and preserved in a new 70% ethanol solution. After dehydration through graded ethanol solution (80, 95 and 100 %), samples were washed with xylene (Bio-Optica, Milano, Italy) and embedded in paraffin (Bio-Optica). Solidified paraffin blocks were cut with a microtome (Leica RM2125 RTS). Sections (5 μm) were stained with Mayer hematoxylin and eosin Y (Merck KGaA; H&E, Darmstadt, Germany) in order to study hepatic parenchyma and intestinal morphology and to measure the perivisceral tissue area or with Alcian blue (Bio-optica) for Alcian blue positive (Ab +) goblet cells detection. Sections were observed using a Zeiss Axio Imager.A2 (Zeiss, Oberkochen, Germany) microscope and images were acquired by a digital camera Axiocam 503 (Zeiss).

To ascertain the degree of hepatic fat accumulation, a quantitative analysis was performed on three section per fish (15 zebrafish larvae per dietary group F1) collected at 50 μm intervals and stained with H&E. The percentage of fat fraction (PFF) was calculated by mean of ImageJ software setting a homogeneous threshold value. Not evaluable areas, such as blood vessels and bile ducts, were not considered. Perivisceral adipose tissue area was measured using ZEN 2.3 software (Zeiss) on three section per fish (15 fish per dietary group) collected at 50 μm intervals and stained with H&E. The semi-quantitative evaluation of histological indexes in the intestine was performed on three transversal sections per fish (15 fish per dietary group; 50 μm intervals) stained with H&E for mucosal folds height and enterocyte supranuclear vacuolization abundance or with Alcian blue for Ab+ goblet cells detection. Specifically, for the morphometric evaluation of mucosal folds height, all the undamaged and non-oblique folds were measured using ZEN 2.3 software (Zeiss). Regarding the semi-quantitative analysis of supranuclear vacuoles and Ab+ goblet cells, an arbitrary unit was assigned as described in Panettieri et al. [**57**]. Scores were assigned as follows: supranuclear vacuoles + = scattered, ++ = abundant; Ab+ goblet cells: + = 0 to 3 per villus, ++ = 4 to 6 per villus, + + + = more than 6 per villus.

Total RNA extraction and cDNA synthesis

Total RNA extraction from five F1 zebrafish larvae collected from each tank at 21 dpf (15 per dietary group) was performed using RNAzol RT reagent (Merck KGaA, Darmstadt, Germany) following the manufacturer's protocol. The final RNA concentration was determined by a NanoPhotometer P-Class (Implen, München, Germany) and the RNA integrity was verified by GelRed^{TM} staining of 28S and 18S ribosomal RNA bands on 1% agarose gel. The cDNA synthesis was performed using the

LunaScript RT SuperMix Kit (New England Biolabs, Ipswich, Massachusetts, USA) using 1 µg of total RNA.

Real-time PCR

PCRs were performed in an iQ5 iCycler thermal cycler (Bio-Rad, Hercules, CA, USA). Reactions were set on a 96-well plate by mixing, for each sample, 1 µL cDNA diluted 1:10, 5 µL of 2x concentrated iQ^{TM} Sybr Green (Bio-Rad) as fluorescent intercalating agent, 0.3 µM of forward primer and 0.3 µM of reverse primer. The thermal profile for all reactions was 3 min at 95 °C and then 45 cycles of 20 s at 95 °C, 20 s at primers specific annealing temperature (for details, please see Table 2), and 20 s at 72 °C. At the end of each cycle, florescence was monitored, and the melting curve analyses showed in all cases one single peak. Relative quantification of the expression of genes involved in fish growth (insulin-like growth factor 1, *igf1*; insulin-like growth factor 2a, *igf2a*; myostatin, *mstnb*), stress response (glucocorticoid receptor, *nr3c1*; heat shock protein 70, *hsp70.1*), long-chain polyunsaturated fatty acids biosynthesis (fatty acid elongase 2, *elovl2*; fatty acid elongase 5, *elovl5*; fatty acid desaturase 2, *fads2*), appetite response (ghrelin, *ghrl*; neuropeptide y, *npy*; cannabinoid receptor 1, *cnr1*; leptin a, *lepa*), immune response (interleukin 1β, *il1b*; interleukin 10,

Table 2. Sequences, Zebrafish Information Network (ZFIN) IDs, and annealing temperature (A.T.) of the primers used for the present study.

Gene	Forward Primer (5'-3')	Reverse Primer (5'-3')	ZFIN IDs	A. T. (°C)
igf1	GGCAAATCTCCACGATCTCTAC	CGGTTTCTCTTGTCTCTCTCAG	ZDB-GENE-010607-2	53
igf2a	GAGTCCCATCCATTCTGTTG	GTGGATTGGGGTTTGATGTG	ZDB-GENE-991111-3	59
mstnb	GGACTGGACTGCGATGAG	GATGGGTGTGGGGATACTTC	ZDB-GENE-990415-165	58
nr3c1	AGACCTTGGTCCCCTTCACT	CGCCTTTAATCATGGGAGAA	ZDB-GENE-050522-503	58
hsp70.1	TGTTCAGTTCTCTGCCGTTG	AAAGCACTGAGGGACGCTAA	ZDB-GENE-990415-91	58
elovl2	CACTGGACGAAGTTGGTGAA	GTTGAGGACACACCACCAGA	ZDB-GENE-011212-1	60
elovl5	TGGATGGGACCGAAATACAT	GTCTCCTCCACTGTGGGTGT	ZDB-GENE-040407-2	60
fads2	CATCACGCTAAACCCAACA	GGGAGGACCAATGAAGAAGA	ZDB-GENE-011212-1	60
ghrl	CAGCATGTTTCTGCTCCTGTG	TCTTCTGCCCACTCTTGGTG	ZDB-GENE-070622-2	58
npy	GTCTGCTTGGGGACTCTCAC	CGGGACTCTGTTTCACCAAT	ZDB-GENE-980526-438	60
cnr1	AGCAAAAGGAGCAACAGGCA	GTTGGTCTGGTACTTTCACTTGAC	ZDB-GENE-040312-3	60
lepa	CTCCAGTGACGAAGGCAACTT	GGGAAGGAGCCGGAAATGT	ZDB-GENE-081001-1	58
il1b	GCTGGGGATGTGGACTTC	GTGGATTGGGGTTTGATGTG	ZDB-GENE-040702-2	54
il10	ATTTGTGGAGGGCTTTCCTT	AGAGCTGTTGGCAGAATGGT	ZDB-GENE-051111-1	56
tnfa	TTGTGGTGGGGTTTGATG	TTGGGGCATTTTATTTTGTAAG	ZDB-GENE-050317-1	53
chia.2	GGTGCTCTGCCACCTTGCCTT	GGCATGGTTGATCATGGCGAAAGC	ZDB-GENE-040426-2014	64
chia.3	TCGACCCTTACCTTTGCACACACCT	ACACCATGATGGAGAACTGTGCCGA	ZDB-GENE-040426-2891	65
arpc1a	CTGAACATCTCGCCCTTCTC	TAGCCGATCTGCAGACACAC	ZDB-GENE-040116-1	60
rpl13	TCTGGAGGACTGTAAGAGGTATGC	AGACGCACAATCTTGAGAGCAG	ZDB-GENE-031007-1	59

il10; tumor necrosis factor α, *tnfa*) and enzymatic hydrolysis of chitin (chitinase 2, *chia.2*; chitinase 3, *chia.3*) was performed. Actin-related protein 2/3 complex, subunit 1A (*arpc1a*) and ribosomal protein, large, 13 (*rpl13*) were used as internal standards in each sample in order to standardize the results by eliminating variation in mRNA and cDNA quantity and quality. Amplification products were sequenced, and homology was verified. No amplification products were observed in negative controls and no primer-dimer formations were observed in the control templates. Data obtained were analysed using the iQ5 optical system software version 2.0 (Bio-Rad) including GeneEx Macro iQ5 Conversion and GeneEx Macro iQ5 files. The same primer sequences designed using Primer3 (starting from zebrafish sequences available in ZFIN) and reported in Zarantoniello et al. [**8**] (chapter 3.1) were used in the present study (Table 2).

Statistical Analyses

All data were analysed by one-way ANOVA, with diet as the explanatory variable. All ANOVA tests were followed by Tukey's post-hoc test. The statistical software package Prism5 (GraphPad Software, 6.01 version) was used. Significance was set at $p < 0.05$ and all the results are presented as mean ± SD.

Results

F1 zebrafish larvae - Growth and survival

Considering SGR% (Fig. 2), no significant differences were detected among experimental groups (22.9 ± 1.0, 22.7 ± 0.9, 23.0 ± 1.1, 22.5 ± 0.8 and 22.7 ± 0.9 % for F1Hi0, F1Hi25, F1Hi50, F1Hi75 and F1Hi100, respectively).

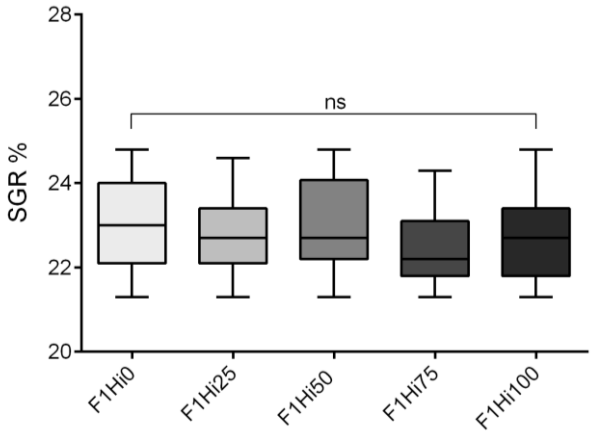


Figure 2. Specific Growth Rate (% weight growth/day) of F1 zebrafish larvae. Boxplots show minimum and maximum (whiskers), first quartile, median and third quartile (box). F1 zebrafish fed diets including 0, 25, 50, 75 and 100 % of BSF prepupae meal respect to FM (Hi0, Hi25, Hi50, Hi75 and Hi100, respectively). ns - no significant differences.

Survival rate did not show significant differences among experimental groups (89 ± 5, 86 ± 6, 85 ± 4, 81 ± 5 and 78 ± 6 % for F1Hi0, F1Hi25, F1Hi50, F1Hi75 and F1Hi100, respectively).

F1 zebrafish larvae - Fatty acids content and composition

The increasing dietary inclusion level of full-fat BSF prepupae meal respect to FM resulted in a significant ($p < 0.05$) increase of SFA (Figure 3a). Fish fed BSF-based diets showed significantly ($p < 0.05$) higher percentages of MUFA and n9 compared to F1Hi0 group (Figure 3a). Regarding PUFA content, F1Hi0 and F1Hi25 groups were characterized by a significantly ($p < 0.05$) higher percentage compared to the other experimental groups, which did not show significant differences among them (Figure 3a). Finally, the increasing inclusion levels of BSF prepupae meal in the experimental diets resulted in a significant ($p < 0.05$) dose-dependent n3 decrease and a parallel slight but significant ($p < 0.05$) dose-dependent n6 increase in F1 zebrafish larvae (Figure 3a). Accordingly, the n6/n3 ratio (Figure 3b) evidenced a significant ($p < 0.05$) increase from F1Hi0 to F1Hi100 groups.

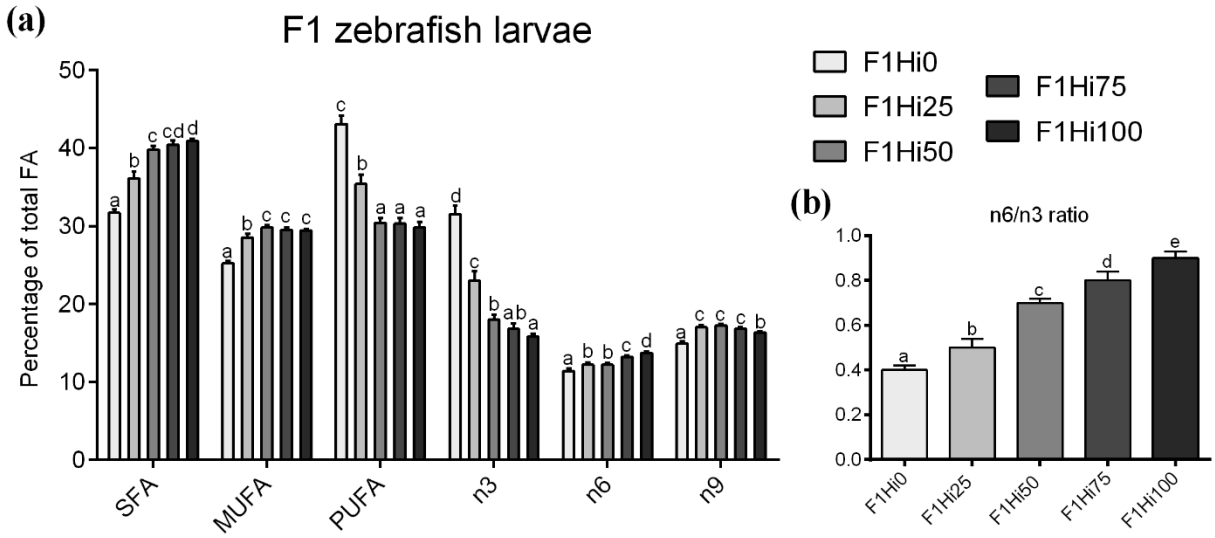


Figure 3. (**a**) Content of SFA, MUFA and PUFA (as % of total FA) and contribution of omega 3 (n3), omega 6 (n6) and omega 9 (n9) fatty acids to lipid profile, and. (**b**) n6/n3 ratio of F1 zebrafish larvae fed the different experimental diets including 0, 25, 50, 75 and 100 % of BSF prepupae meal respect to FM (Hi0, Hi25, Hi50, Hi75 and Hi100, respectively). Different letters indicate statistically significant differences among experimental groups compared within the same fatty acid class ($p < 0.05$). Values are presented as mean ± SD (n = 12).

Considering the fatty acids composition of F1 zebrafish larvae (Table 3), the most represented SFA in all the dietary treatments was palmitic acid (16:0), followed by stearic (18:0) and myristic (14:0) acids. The percentage of both palmitic and myristic acids was significantly ($p < 0.05$) lower in F1Hi0 group compared to F1Hi50, FiHi75 and F1Hi100 ones, which did not evidence significant differences among them. In addition, the percentage of lauric acid (12:0) significantly ($p < 0.05$) increased according to the increasing BSF prepupae meal dietary inclusion respect to FM. With regard to

MUFA, the predominant fatty acid in all the dietary treatments was oleic acid (18:1n9), which was significantly ($p < 0.05$) higher in all the groups fed BSF-based diets compared to F1Hi0. The increasing dietary BSF prepupae meal level respect to FM resulted in a significant ($p < 0.05$) increase in both 7-hexadecenoic (16:1n9) and vaccenic (18:1n7) acids percentages, while no significant differences were detected among experimental groups considering palmitoleic acid (16:1n7).

Table 3. Fatty acids composition (as % of total fatty acids) and DHA/EPA ratio of F1 zebrafish larvae.

	F1Hi0	**F1Hi25**	**F1Hi50**	**F1Hi75**	**F1Hi100**
10:0	0.010±0.001	0.023±0.001	0.047±0.004	0.052±0.003	0.080±0.009
12:0	0.29±0.01 ^{a}	2.70±0.10 ^{b}	4.80±0.30 ^{c}	5.70±0.30 ^{d}	6.40±0.50 ^{e}
13:0	0.051±0.002	0.067±0.002	0.084±0.005	0.088±0.002	0.092±0.008
14:0	4.5±0.3 ^{a}	5.2±0.3 ^{ab}	5.5±0.5 ^{b}	5.4±0.5 ^{b}	5.5±0.5 ^{b}
14:1n5	0.09±0.01	0.17±0.02	0.28±0.03	0.29±0.03	0.26±0.03
15:0	0.78±0.02	0.90±0.03	0.98±0.04	0.98±0.06	0.99±0.07
16:0	18.2±0.8 ^{a}	19.6±1.0 ^{ab}	20.3±1.0 ^{b}	20.2±0.7 ^{b}	19.9±0.9 ^{b}
16:1n9	0.90±0.05 ^{a}	1.10±0.04 ^{b}	1.40±0.10 ^{c}	1.30±0.10 ^{c}	1.30±0.10 ^{c}
16:1n7	7.0±0.5 ^{a}	7.5±0.4 ^{a}	7.5±0.6 ^{a}	7.2±0.6 ^{a}	7.2±0.5 ^{a}
16:2n7	0.19±0.02	0.20±0.01	0.22±0.02	0.29±0.03	0.28±0.03
17:0	0.80±0.04 ^{a}	0.90±0.05 ^{a}	1.20±0.05 ^{b}	1.30±0.07 ^{b}	1.20±0.09 ^{b}
17:1n7	0.09±0.01	0.29±0.02	0.46±0.04	0.54±0.04	0.54±0.04
18:0	6.6±0.3 ^{a}	6.2±0.4 ^{a}	6.3±0.5 ^{a}	6.1±0.5 ^{a}	6.1±0.4 ^{a}
18:1n9	12.4±0.6 ^{a}	14.4±1.0 ^{b}	14.9±1.0 ^{b}	14.7±1.2 ^{b}	14.6±1.1^{b}
18:1n7	3.1±0.2 ^{a}	3.6±0.2 ^{a}	4.4±0.3 ^{b}	4.7±0.4 ^{bc}	5.1±0.4 ^{c}
18:2n6	8.7±0.6 ^{a}	9.0±0.5 ^{a}	8.1±0.6 ^{a}	8.8±0.7 ^{a}	9.0±0.6 ^{a}
18:3n3	1.3±0.1 ^{a}	1.3±0.1 ^{a}	1.7±0.1 ^{b}	1.9±0.2 ^{b}	1.7±0.2 ^{b}
20:0	0.32±0.02	0.30±0.02	0.28±0.02	0.29±0.02	0.33±0.03
20:1n9	1.06±0.06	1.01±0.07	0.68±0.04	0.60±0.04	0.40±0.03
20:2n6	0.31±0.03	0.30±0.02	0.30±0.03	0.37±0.04	0.37±0.03
20:3n6	0.42±0.04	0.56±0.05	0.82±0.08	0.92±0.07	1.00±0.10
20:4n6	2.0±0.1 ^{a}	2.3±0.1 ^{a}	2.9±0.2 ^{b}	3.1±0.2 ^{bc}	3.3±0.2 ^{c}
20:3n3	0.11±0.01	0.10±0.01	0.11±0.01	0.10±0.01	0.11±0.01
20:5n3	8.6±0.5 ^{c}	5.0±0.4 ^{b}	3.1±0.3 ^{a}	2.8±0.3 ^{a}	2.7±0.2 ^{a}
22:0	0.17±0.02 ^{c}	0.24±0.03 ^{b}	0.30±0.03 ^{a}	0.25±0.03 ^{a}	0.28±0.03 ^{a}
22:1n9	0.48±0.05	0.47±0.04	0.21±0.03	0.15±0.03	0.03±0.01
22:6n3	21.5±1.0 ^{c}	16.6±1.1 ^{b}	13.1±1.0 ^{a}	12.0±0.9 ^{a}	11.3±0.8 ^{a}
DHA/EPA	2.5±0.2 ^{a}	3.3±0.4 ^{b}	4.2±0.5 ^{c}	4.3±0.5 ^{c}	4.2±0.4 ^{c}

Means within rows bearing different letters are significantly different ($p<0.05$). Values are reported as mean ± SD ($n = 9$). Statistical analysis was performed only for fatty acids > 1%. fatty acids with a percentage < 1% were excluded from any statistical analyses because their concentrations were close to the limit of detection. F1 zebrafish fed diets including 0, 25, 50, 75 and 100 % of BSF prepupae meal respect to FM (Hi0, Hi25, Hi50, Hi75 and Hi100, respectively).

Finally, docosahexaenoic (22:6n3, DHA) and linoleic (18:2n6) acids represented the most abundant PUFA in all the dietary treatments. In particular, linoleic acid levels did not show significant differences among the experimental groups. Conversely, the increasing dietary inclusion level of BSF prepupae meal respect to FM resulted in a significant ($p < 0.05$) increase in α-linolenic (18:3n3) and arachidonic (20:4n6) acids percentages and in a significant ($p < 0.05$) decrease in DHA and eicosapentaenoic acid (20:5n3, EPA) percentages. Considering the DHA/EPA ratio, F1Hi0 and F1Hi25 groups were characterized by significantly ($p < 0.05$) lower values compared to the other experimental groups which did not evidence significant differences among them.

F1 zebrafish larvae - Histology

With respect to the liver, histological analyses were performed to evaluate lipid accumulation or steatosis. Results evidenced a modest fat liver parenchyma with a widespread presence of hepatocytes with cytoplasm filled with fat, interspersed with normal hepatocytes in all the experimental groups highlighting a similar degree of lipid accumulation (Figure 4a-e). These results were confirmed by PFF quantification, which did not show significant differences among the experimental groups (Figure 4f). No significant differences were detected among the experimental groups even with regard to the perivisceral adipose tissue area (Figure 4g).

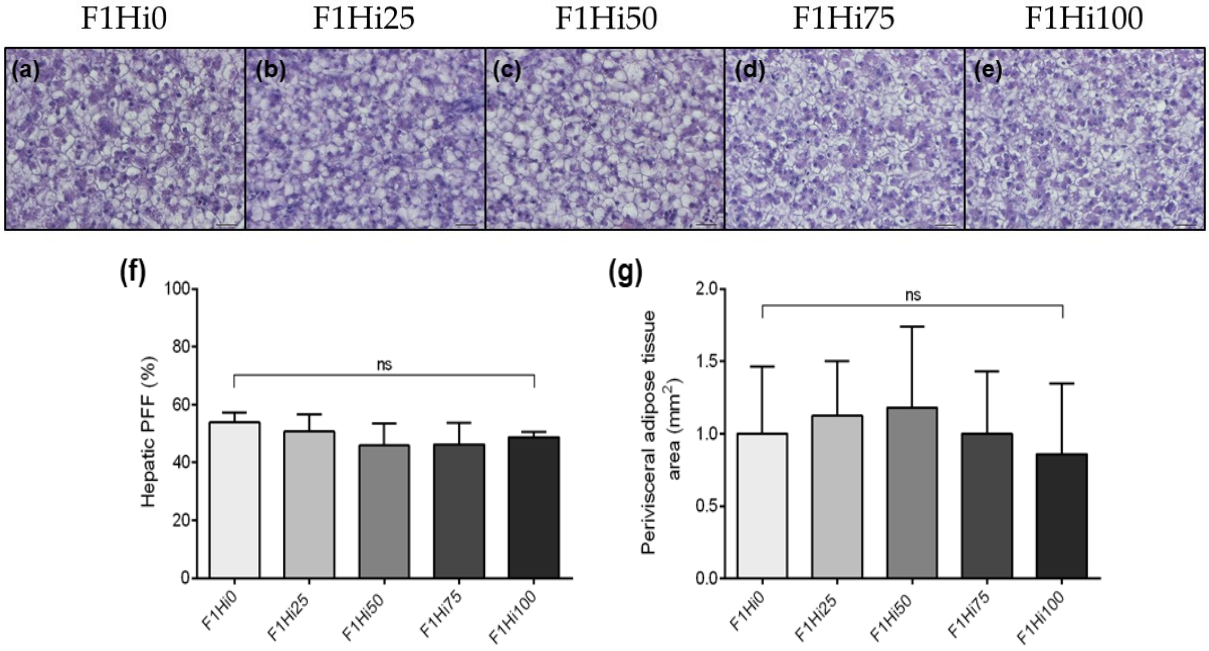
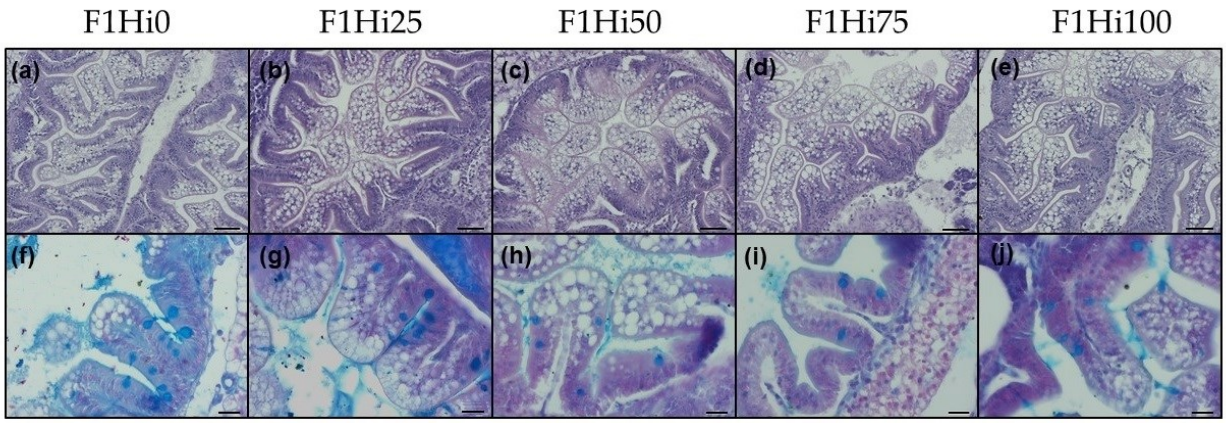


Figure 4. (**a**-**e**) Example of hepatic parenchyma histomorphology, (**f**) percentage of fat fraction (PFF) in liver tissue and (**g**) perivisceral adipose tissue area (mm^2) of F1 zebrafish larvae fed diets including 0, 25, 50, 75 and 100 % of BSF prepupae meal respect to FM (F1Hi0, F1Hi25, F1Hi50, F1Hi75 and F1Hi100 groups). Scale bars: 20 µm. For PFF and perivisceral adipose tissue area, values are shown as mean ± standard deviation (n = 15). ns: no significant differences.

Finally, with regard to medium intestine (Figure 5a-j), no morphological alterations or signs of inflammation were evident in any of the experimental groups. In addition, no significant differences were observed among the experimental groups in terms of mucosal folds length and supranuclear vacuoles and Ab+ goblet cell abundance (Figure 5k).



(k)

	F1Hi0	F1Hi25	F1Hi50	F1Hi75	F1Hi100
Mucosal folds length (µm)	113.5±17.3	110.0±6.9	112.4±15.1	105.8±10.5	114.9±9.1
Supranuclear vacuoles	++	++	++	++	++
Ab+ goblet cells	++	++	++	++	++

Figure 5. (**a-j**) Example of medium intestine histomorphology and (**k**) histological indexes (mucosal folds length, supranuclear vacuoles and Ab+ goblet cell abundance) measured in this gut tract of F1 zebrafish larvae fed diets including 0, 25, 50, 75 and 100 % of BSF prepupae meal respect to FM (F1Hi0, F1Hi25, F1Hi50, F1Hi75 and F1Hi100 groups). Scale bars and staining: (**a-e**) 50 µm, H&E; (**f-j**) 20 µm, Ab. Letters: gc = Ab+ goblet cells. For histological indexes (**k**), values of mucosal folds length are shown as mean ± SD (n = 15). Scores: supranuclear vacuoles + = scattered, ++ = abundant; Ab+ goblet cells + = 0 to 3 per villus, ++ = 4 to 6 per villus, +++ = more than 6 per villus. No significant differences were detected among the experimental groups.

F1 zebrafish larvae - Real-time PCR results

Growth factors. Considering *igf1*, *igf2a* and *mstnb* gene expression (Figure 6a-c), no significant differences were detected among experimental groups.

Stress response. Regarding genes involved in the stress response (*nr3c1* and *hsp70.1*; Figure 6d,e), groups fed the lowest BSF prepupae meal inclusion levels respect to FM (F1Hi0 and F1Hi25) showed a significant ($p < 0.05$) upregulation compared to F1Hi50, F1Hi75 and F1Hi100 groups, which did not evidence significant differences among them.

Lipid metabolism. Considering *elovl2* and *elovl5* gene expression (Figure 6f,g), F1Hi100 group showed the highest gene expression ($p < 0.05$) with respect to the other experimental groups, while no significant differences were observed among them (except for *elovl2* gene expression which was significantly downregulated in F1Hi50 compared to F1Hi75; $p < 0.05$). With regard to *fads2* gene expression (Figure 6h), all groups fed on BSF-based diets showed a significant ($p < 0.05$) upregulation compared to F1Hi0 group.

Appetite. Regarding gene expression of *ghrl*, *npy* and *cnr1* (Figure 6i-k), the experimental groups fed BSF-based diets showed a significantly ($p < 0.05$) downregulation with respect to F1Hi0 group. With regard to *lepa* gene expression (Figure 6l), a significantly ($p < 0.05$) BSF dose-dependent decreasing trend was evident among the experimental groups, with F1Hi0 that was characterized by a significant upregulation compared to F1Hi75 and F1 Hi100 groups.

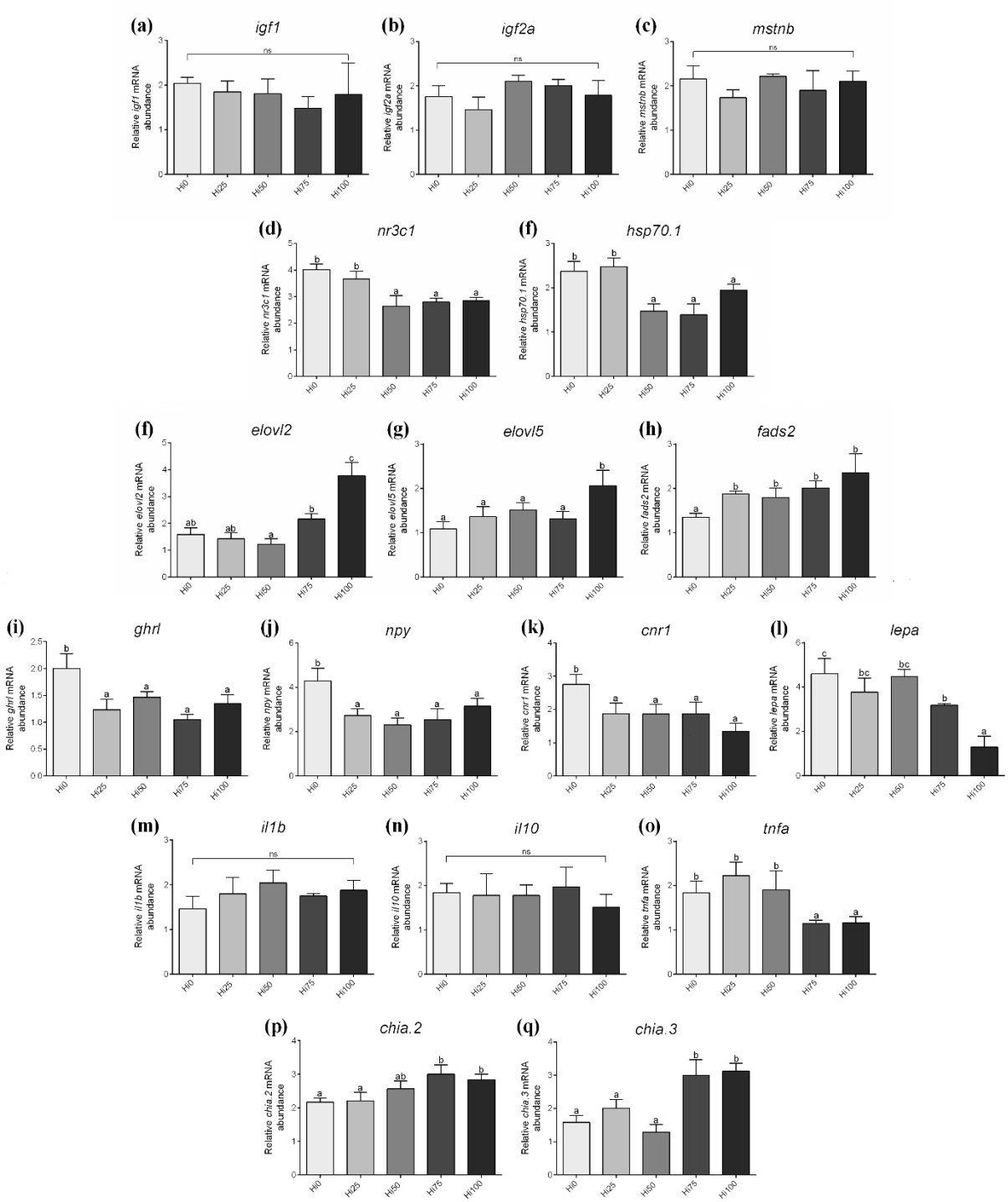


Figure 6. Relative mRNA abundance of genes analysed in F1 zebrafish larvae. (**a**) *igf1*, (**b**) *igf2a*, (**c**) *mstnb*, (**d**) *nr3c1*, (**e**) *hsp70.1*, (**f**) *elovl2*, (**g**) *elovl5*, (**h**) *fads2*, (**i**) *ghrl*, (**j**) *npy*, (**k**) *cnr1*, (**l**) *lepa*, (**m**) *il1b*, (**n**) *il10*, (**o**) *tnfa*, (**p**) *chia.2* and (**q**) *chia.3*. Different letters specify statistically significant differences among experimental groups ($p < 0.05$). Values are shown as mean ± standard deviation (n = 5). ns: no significant differences. F1 zebrafish fed diets including 0, 25, 50, 75 and 100 % of BSF prepupae meal respect to FM (Hi0, Hi25, Hi50, Hi75 and Hi100, respectively).

Immune response. Considering *il1b* and *il10* gene expression (Figure 6m,n), no significant differences were observed among experimental groups. Differently, groups fed the highest BSF inclusion levels respect to FM (F1Hi75 and F1Hi100) showed a significant ($p < 0.05$) downregulation in *tnfa* gene expression (Figure 6o) compared to F1Hi0, F1Hi25 and F1Hi50 groups that did not evidence significant differences among them.

Chitinase. Regarding *chia.2* and *chia.3* gene expression (Figure 6p,q), F1Hi75 and F1Hi100 groups were characterized by a significant ($p < 0.05$) upregulation compared to the other groups which did not show significant differences among them (except for *chia.2* gene expression in F1Hi50 group).

Discussion

Nutritional programming covers the metabolic adaptations to a dietary stimulus applied in pre- or post-natal stages that persist later in life, possibly improving health and survival [**58-60**]. In this way, parental diet may have effects on the offspring, especially during the early developmental stages characterized by organogenesis, establishment of metabolic pathways and high metabolic plasticity [**27**]. In light of FM and FO substitution with more sustainable aquafeed ingredients, several studies have been published highlighting the possibility to nutritionally programming fish offspring via broodstock nutrition to plant-based diets [**36**,**38**,**61-63**]; however, no studies have been performed using insect-based diets.

The present study aimed to evaluate the possible cross-generation effects of BSF-based diets on F1 larval development using zebrafish as an experimental model. Results are discussed with a comparison to F0 zebrafish larvae that were fed on the same BSF-based diets used in the present study, but that were not nutritionally programmed through parental feeding, as reported in Zarantoniello et al. [**8**] (chapter 3.1). The experimental diets used in in the present study, as well as in Zarantoniello et al. [**8**] (chapter 3.1), were formulated to be iso-nitrogenous and iso-lipidic, despite a progressive reduction of marine resources and a parallel increase of BSF prepupae meal. To maintain the dietary protein and lipid intake at a constant level, some vegetable ingredients were included. To relate all the results obtained to the dietary BSF prepupae meal inclusion with respect to FM, wheat flour and a mixture of highly digestible wheat gluten and pea protein concentrates were used. In this regard, wheat flour is a common dietary filler due to its low nutritional value [**64**,**65**]. Furthermore, wheat- and pea protein-based diets have already been demonstrated to not affect zebrafish growth and gene expression compared to a control diet based on FM [**66**].

In recent years, it has been demonstrated that a nutritional stimulus during broodstock reproduction, represented by up to 70% of FO dietary replacement by a combination of vegetable oils, was able to promote growth performance in gilthead seabream offspring juveniles [**36-38**,**67**]. Accordingly, the

BSF-based diets used in the present study have been shown to improve zebrafish SGR% and to upregulate *igfs* gene expression in F0Hi50, F0Hi75 and F0Hi100 groups compared to a control group (F0Hi0) [**8**] (chapter 3.1). Conversely, the F1 zebrafish larvae analysed in the present study did not show these differences. This result could be related to the fact that feeding broodstock with experimental diets characterized by different dietary fatty acids profiles can markedly affect offspring lipid metabolism, with particular emphasis on highly energy demanding PUFA biosynthesis [**27**,**36**,**67**,**68**]. PUFA delivered from parental diet are considered regulators of embryonic gene expression [**33**,**69**,**70**]. As reported by Zarantoniello et al. [**8**] (chapter 3.1), in F0 zebrafish larvae, an increasing dietary BSF prepupae meal dietary inclusion respect to FM resulted in a parallel decrease in their PUFA content. In contrast, in the present study, F1 zebrafish larvae from F1Hi50, F1Hi75 and F1Hi100 groups showed a lower PUFA content respect to F1Hi0 and F1Hi25, but they did not evidence significant differences among them. Several studies on farmed fish species reported that providing diets poor in long-chain PUFA to broodstock enhanced offspring ability to synthesize these fundamental fatty acids, as denoted by the upregulation of genes (like elovl5 and fads2) involved in this process [**36-38**,**61**,**62**,**71**]. In particular, fads.2 codifies the D6-desaturase enzyme, a strong marker for document nutritional programming effects from broodstock to offspring, since it is considered the rate limiting step in long-chain PUFA biosynthesis [**72-75**]. As reported in Zarantoniello et al. [**8**] (chapter 3.1), only the zebrafish larvae fed the highest BSF prepupae meal dietary inclusion respect to FM (F0Hi100) showed a significant *elovl5* and *fads2* upregulation compared to the other experimental groups. In the present study, this trend was evident for both *elovl2* and *elovl5*, but not for *fads2*, expression of which, differently from the F0 study, was significantly higher in all the groups fed BSF-based diets. This upregulation can be correlated with the reduction of differences in PUFA content among zebrafish larvae from F1Hi50, F1Hi75 and F1Hi100 groups.
Furthermore, nutritional programming, besides acting on genes involved in lipid metabolism that promote a better use of low FM and FO diets by the offspring, can reduce the risk to develop hepatic steatosis, often evidenced in fish fed both plant- and BSF-based diets [**15**,**16**,**37**]. In this regard, Zarantoniello et al. [**8**] (chapter 3.1) reported that F0 zebrafish larvae fed the highest BSF dietary inclusion levels (75 and 100 %) were characterized by a severe condition of hepatic steatosis that, in turn, was addressed as the potential cause of the overexpression of stress markers (*nr3c1* and *hsp70.1*). Conversely, in the present study, the histological analyses of F1 zebrafish larvae revealed no signs of hepatic steatosis. The same degree of hepatic lipid accumulation was evident among all the experimental groups, despite a dietary BSF dose-dependent increase in n6/n3 ratio that was previously related to steatosis onset [**8**,**16**]. Accordingly, the PFF analyses did not show significant differences among the F1 experimental groups, which all evidenced lower values compared to

F0Hi75 and F0Hi100 [**8**] (chapter 3.1). Furthermore, the expression of genes involved in the stress response (*nr3c1* and *hsp70.1*) was lower in F1Hi50, F1Hi75 and F1Hi100 groups compared to the other ones. These results are in accord with a previous study in which it was demonstrated that stress-related genes can also be modulated in nutritionally programmed offspring from broodstock fed diets with increased substitution of FO with linseed oil [**38**]. Furthermore, the downregulation of stress markers in F1Hi75 and F1Hi100 could explain the lower leptin (*lepa*) gene expression detected in these groups. In fish, increased cortisol levels result in a synergic increase in hepatic leptin mRNA levels due to the necessity to mobilize energy reserves in response to a stress condition [**76**,**77**]. Conversely, this correlation cannot be applicable to F1Hi50 group, which showed a high *lepa* gene expression despite a downregulation of stress markers. However, F1 zebrafish larvae from F1Hi50 were characterized by the highest (even if not significantly) perivisceral adipose tissue area, which could explain the *lepa* gene expression of this group, since the amount of adipose tissue is positively correlated with the circulating leptin levels [**78**,**79**].

Considering the orexigenic signals analysed in the present study, all the groups fed BSF-based diets showed a significant *ghrl*, *npy* and *cnr1* downregulation compared to F1Hi0. These results are in line with the biometric ones and with a previous study in which nutritionally programmed zebrafish were found to be in a satiated state compared to the control groups [**80**]. Conversely, the higher orexigenic signals gene expression found in all F0 zebrafish larvae fed on BSF-based diets was related to a compensatory mechanism that increased food intake with a consequent faster growth rate, possibly in relation to dietary deficiency of important nutrients, like DHA [**8**,**30**].

Nutritional programming may make possible to obtain fish better adapted to use specific dietary ingredients, also by acting on the gastrointestinal tract which, in fish, is able to adapt to rapid shifts in environmental conditions, including diet [**80-82**]. In the present study, no specific inflammatory events and no differences in histopathological indexes were detected through the histological analyses on F1 zebrafish larvae intestine from any of the dietary treatments. The absence of negative effects on gut health was also observed in F0 zebrafish larvae, which, however, presented an upregulation of molecular markers involved in the immune response when fed 50, 75 and 100 % BSF inclusion levels respect to FM, possibly suggesting future development of inflammation [**8**]. Conversely, in the present study, neither *il1b* nor *il10* gene expression showed significant differences among the experimental groups, and *tnfa* was downregulated in F1Hi75 and F1Hi100. Accordingly, it was demonstrated that both pro- and anti-inflammatory cytokines gene expression can be positively programmed by early nutrition in zebrafish juveniles to better face a dietary challenge later in life [**83**]. No differences in proinflammatory cytokine gene expression were evidenced also in adult zebrafish fed BSF-based diets (0, 25 and 50 % with respect to FM) over the whole life cycle [**15**]

(chapter 2.1). The positive effects of BSF-based diets on gut health can be attributed to the properties of lauric acid (12:0) and chitin, which have been addressed as immune-boosting molecules [**3**,**84**]. The long-term experience with BSF-based diets, potentially also across generations through nutritional programming, could led to a more extended effect of these BSF dietary components, resulting both in the absence of visible inflammatory events in the intestine and to a positive modulation of the molecular markers involved in the immune response. Accordingly, the chitinases (*chia.2* and *chia.3*) upregulation in F1Hi75 and F1Hi100 groups could have possibly increased chitin digestion enhancing its use as prebiotic, having a positive effect on gut microbial communities and, thus, on overall gut health [**85-87**].

The present study highlighted that nutritional programming through broodstock feeding can have positive effects on the offspring when insects are included in the diets. The results demonstrated that, using nutritional programming, the FM substitution level with BSF prepupae meal in the diet can be extended by up to almost 100% during zebrafish larval development without negative effects on fish growth and welfare. Nutritional programming should thus be considered as one of the potential solutions for counteracting the recurring negative side effects of high BSF prepupae meal dietary inclusion levels. The results obtained in the present study, which used the experimental model zebrafish, may represent a starting point for their application to finfish culture.

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Chapter 4

Practical BSF-based diets intended for farmed fish species

4.1

Physiological responses of Siberian sturgeon (*Acipenser baerii*) juveniles fed on full-fat insect-based diet in an aquaponic system

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Abstract

Over the last years, the potential use of Black Soldier Fly (BSF) meal as a new and sustainable aquafeed ingredient has been largely explored in several fish species. However, only fragmentary information is available about the use of BSF meal-based diets in sturgeon nutrition. In consideration of a circular economy concept and a more sustainable aquaculture development, the present research represents the first comprehensive multidisciplinary study on the physiological effects of a BSF-based diet during sturgeon culture in an aquaponic system. Siberian sturgeon (*Acipenser baerii*) juveniles were fed over a 60-day feeding trial on a control diet (Hi0) and a diet containing 50% of full-fat BSF meal respect to fish meal (Hi50). Physiological responses of fish were investigated using several analytical approaches, such as gas chromatography-mass spectrometry, histology, Fourier Transformed Infrared Spectroscopy (FTIR), microbiome sequencing and real-time PCR. While aquaponic systems performed optimally during the trial, Hi50 group fish showed lower diet acceptance that resulted in growth and survival reduction, a decrease in hepatic lipids and glycogen content (FTIR), a higher hepatic *hsp70.1* gene expression and a worsening in gut histological morphometric parameters. The low feed acceptance showed by Hi50 group sturgeon highlighted the necessity to improve the palatability of BSF-based diet designed for sturgeon culture.

Introduction

Siberian sturgeon (*Acipenser baerii*) is one of the most valuable species in aquaculture, due to the production of caviar and high-quality meat for human consumption. Restocking programs are also of great interest for this species since it is included in the list of endangered wild populations [**1**]. Compared to other sturgeon species, *Acipenser baerii* shows rapid growth rate, resistance to pathogens, a relatively short reproductive cycle (7-8 years) and can be reared using a wide range of diets and environmental conditions [**2,3**]. Owing to its features and high commercial value, intensive aquaculture of Siberian sturgeon is presently carried out in different parts of the world and, in recent years, several studies regarding farming-related biology [**4**] and nutritional requirements [**1**,**5**] have been performed. To ensure proper growth and constant productivity, aquafeeds commonly used in sturgeon rearing are mainly represented by high-energy diets largely based on fish meal (FM) and fish oil (FO) to meet proper protein and n3 highly unsaturated fatty acids requirement [**6**,**7**]. However, for a more sustainable aquaculture development, the use of FM and FO should be limited for both ecological and economic issues [**8**]. For this reason, the discovery of novel nutritious and more sustainable ingredients for aquafeeds formulation is crucial [**9**].

Over the last years, several ingredients have been evaluated as FM substitutes in sturgeon aquaculture, ranging from vegetable ones like soybean meal [**5**], rice concentrate [**10**], corn gluten [**11**], and the

cyanobacterium *Arthrospira platensis* (Spirulina) [**12**] to animal ones such as poultry by-products [**7**]. Nowadays, with the goal of a further reduction of aquaculture environmental footprint, insect species like the Black Soldier Fly (*Hermetia illucens*; BSF) represent very promising candidates as FM alternatives [**13**]. The great interest in the BSF meal as aquafeed ingredient is due to their eco-friendly rearing in terms of land use, water consumption, CO_2 emissions and on high feed conversion efficiency (BSF larvae are able to grow on low value organic by-products converting them into valuable biomass) [**14**,**15**]. Furthermore, insects like Diptera and Coleoptera are part of the natural diet of Siberian sturgeon [**16**]. It is well known that these insects possess bioactive compounds like chitin, which at certain concentrations are able to boost the fish immune system and promote gut microbiota diversification [**17**,**18**]. From the nutritional point of view, BSF prepupae meal have a suitable protein content, and the amino acids composition is similar to that of FM [**19**]. However, its fatty acids profile present some disadvantages, such as a high content of saturated fatty acids (SFA) and an extremely low content in polyunsaturated (PUFA) ones [**20**]. PUFA are particularly important for fish since deficiencies in these compounds may cause a general deterioration of fish health, poor growth, low feed efficiency and often high mortality [**21-23**]. Previous studies demonstrated that a proper PUFA dietary content is essential to sustain both larval and adult Siberian sturgeon growth and welfare [**24**,**25**]; these compounds play a pivotal role in sturgeon fillet and caviar quality [**26**].

Some recent studies tested different defatted BSF meal inclusion levels in aquafeed formulation for several fish species but results on fish physiological responses are still controversial [**27-30**]. This topic, however, has scarcely been investigated in sturgeon aquaculture and most of the results are limited to zootechnical analyses [**31-33**]. Nowadays, several laboratory approaches (histology, molecular biology, gas chromatography and infrared spectroscopy) are available to evaluate fish welfare and quality and represent valid tools to assess the inclusion of new ingredients, like insect meal, in aquafeed production [**23**,**34-36**]. In addition, the use of full-fat BSF meal is preferable to the highly defatted in order to reduce manufacturing costs [**37**,**38**]. This aspect has been recently addressed by Truzzi et al. [**39**]. These authors developed an enrichment procedure to increase insects' PUFA content that allowed to include up to 50% of BSF prepupae meal compared to FM in zebrafish diet without impairing fish growth and welfare [**38**]. Because of this positive result, the same enriched full-fat BSF dietary inclusion percentage was chosen for the present study, expecting to obtain more promising results respect to Caimi et al. [**32**] that evidenced a significant reduction of feed consumption and growth performance in Siberian sturgeon juveniles fed on a diet in which FM was 50% replaced with highly defatted BSF larvae meal.

In the present study, Siberian sturgeon juveniles were fed over a 60-days feeding trial on a control diet (based on FM and FO; Hi0) and a diet containing 50% of enriched BSF prepupae meal respect

to FM (Hi50). Results obtained from zootechnical performances, fillet fatty acids composition, liver and gut integrity, expression of genes involved in fish growth, stress and immune response and gut microbiome represent the first multidisciplinary investigation on the physiological effects of BSF-based diets in sturgeon juveniles. Furthermore, this is the first feeding trial using insect-based diets performed in an aquaponics system. This green technology combines aquaculture (production of fish) with horticulture (vegetables production) saving energy, water and nutrients [**40**], representing an important step for the development of a sustainable aquaculture in a future zero-waste generation [**41**,**42**].

Methods

Ethics

All zootechnical trials were conducted in agreement with the Italian legislation on experimental animals and were approved by the Ethics Committee of the Università Politecnica delle Marche (Aut. No. 01/2019)

Insects rearing and fish diet production

Insects were reared on coffee by-product (coffee silverskin) obtained from the roasting process at Saccaria Caffè SRL, Marina di Montemarciano, Italy. Coffee silverskin was processed as already described by Zarantoniello et al. [**38**] (chapter 3.1) and the substrate was enriched with a 10% (w/w) of *Schizochytrium* sp [**39**]. Six days old larvae (purchased from Smart Bugs s.s. Ponzano Veneto, Treviso, Italy) were divided in groups of 640 larvae per replicate (n = 65) for a total of 41˙600 specimens. Each replicate consisted of a plastic box (57 × 38 × 16 cm) screened with fine-mesh cotton gauze and covered with a lid provided with 90 ventilation holes (0.05 cm diameter) [**14**]. Each box was provided with a feeding rate of 448 g once a week. Insects were reared in a climatic chamber at 27 ± 1 °C with 65 ± 5 % of relative humidity [**14**]. Prepupae, identified by the change in tegument colour, were collected, washed, dried and stored at -80 °C.

BSF full-fat prepupae were freeze-dried and grounded in a mill (Retsch Centrifugal Grinding Mill ZM 1000; Retsch GmbH, Germany) to obtain the insect meal for subsequent feed formulation. Two experimental diets were prepared: (i) control diet (Hi0) based on marine (FM and FO) and vegetable protein (pea protein concentrate and wheat gluten meal) resources; (ii) BSF-based diet (Hi50), obtained from the Hi0 formulation including 50% of BSF prepupae meal in substitution of marine resources (both FM and FO). For diet production, all grounded ingredients (0.5 mm) were well mixed and then FO and/or water were added to form a moist blend (Kenwood kMix KMX53 stand Mixer). The blend was pelleted with a grinder (using a 3 mm die meat) and pellets were dried in an oven at

40 °C for 48 h. Dried pellets were crushed and sieved through a battery of sieves to obtain feed particles ranging from 0.5 to 1.0 mm in diameter. The obtained diets were stored in under vacuum bags and kept at -20 °C until use. Feed samples were analysed for dry matter, crude protein, and ash according to AOAC International [**43**]. The total lipid fraction (crude lipid) was determined according to the Bligh and Dyer method as previously described in Burja et al. [**44**]. The gross energy content was determined using an adiabatic calorimetric bomb (IKA C7000, Werke GmbH & Co., Staufen, Germany). Diets ingredient and proximate composition are shown in Table 1.

Table 1. Ingredients (g/kg) and proximate composition (% as feed basis) of experimental diets.

	Hi0	**Hi50**
Ingredients (g/kg)		
Fish meal [1]	395	198
Pea protein concentrate [2]	120	120
BSF prepupae meal	-	230
Wheat gluten meal [1]	130	204
Wheat flour [1]	258	183
Fish oil [1]	65	33
Soy lecitin [1]	8	8
Mineral [3] & Vitamin [4] supplement	14	14
Binder [3]	10	10
Proximate composition (%)		
Dry Matter; DM	94.5	93.3
Crude protein; CP	48.5	50.0
Crude lipid; CL	11.3	9.8
Ash	8.1	9.2
Gross Energy (Mj/kg)	19.9	19.2

[1] By Skretting Italia, Mozzecane VR (Italy); **[2]** Lombarda trading srl, Casalbuttano & Uniti (CR, Italy); [3] Sodium alginate (Merck KGaA, Darmstadt, Germany). [3] Mineral supplement composition (% mix): $CaHPO_4*2H_2O$. 78.9; MgO. 2.725 g; KCl. 0.005; NaCl. 17.65; FeCO3. 0.335; $ZnSO_4*H_2O$. 0.197; $MnSO_4*H2O$. 0.094; $CuSO_4*5H_2O$. 0.027; Na_2SeO_3. 0.067. **[4]** Vitamin supplement composition (% mix): thiamine HCL Vit B1. 0.16; riboflavin Vit B2. 0.39; pyridoxine HCL Vit B6, 0.21; cyanocobalamine B12, 0.21; niacin Vit PP, 2.12; calcium pantotenate, 0.63; folic acid, 0.10; biotin Vit H, 1.05; myoinositol, 3.15; stay C Roche, 4.51; tocoferol Vit E, 3.15; menadione Vit K3, 0.24; Vit A (2500UI/kg diet) 0.026; Vit D3 (2400UI/kg diet) 0.05; choline chloride, 83.99.

Fish, aquaponic system and experimental design

The 60-day feeding trial was conducted at the aquaponics facility "Cooperativa Agricola Tanto Sole" (Treia, Macerata, Italy). Juvenile Siberian sturgeons, purchased from Azienda Agricola Pisani Dossi s.s., Cisliano, Milano, Italy), were acclimated for 1 week in a single 500 L tank equipped with mechanical, biological and UV filtration (Panaque, Viterbo, Italy). Tank temperature was 18 ± 0.5 °C; ammonia (NH_3) and nitrite (NO_2^-) were < 0.05 mg/L and nitrate (NO_3^-) 10 mg/L, according to sturgeon rearing requirements [**45**]. At the end of the acclimation period, fish were randomly allocated into six Media Based Aquaponic Systems (80 specimens per tank). Each aquaponic system consisted

of a 1.56 m^2 hydroponic unit for plants cultivation and a 600 L fish tank, for a total volume of 720 L of water.

Fish unit. The six systems were maintained at constant temperature (18.0 ± 0.5 °C) by chillers TK500 (Teco, Ravenna, Italy). Evaporated water was replaced on request and the systems were subjected to a natural photoperiod (11 L / 13 D). Water samples were weekly collected in order to test ammonia, nitrite, nitrate, and phosphate (PO_4^{3-}) using Hanna reagents and a HI83399 spectrophotometer (Hanna instruments, Villafranca Padovana, Italy).

Hydroponic unit. Each hydroponic unit was filled with expanded clay with biological and mechanical filtration function [**40**], necessary to guarantee a physical support for plant growth. Specifically, in each hydroponic unit, 16 lettuce (*Lactuca sativa*; initial weight: 2.95 ± 0.5 g) and 3 celery (*Apium graveolens*; initial weight: 20.8 ± 5.0 g) seedlings were planted two days before introduction of the fish (density = 12 plants/m^2). Recirculating water flow from the fish tank to the hydroponic unit was regulated by a 1900 L/h pump (Eheim GmbH & Co, Deizisau, Germany) completing 3 water renewals per hour. Specifically, water was pumped from the fish tank to the hydroponic unit, and then returned to the fish unit through a siphon. The siphon was equipped with further synthetic foam for extra mechanical filtration (foam was cleaned once per week).

Feeding trial. At the beginning of the experiment (t_0), the six aquaponic systems were randomly assigned to the experimental groups (Hi0, Hi50) according to an experimental design with triplicate tanks per dietary treatment. Feeding trial duration was 60 days, in which sturgeons almost triplicated their weight and were fed as follows: fish fed the control diet (Hi0 group); fish fed the diet that included 50% of BSF full-fat prepupae meal respect to FM (Hi50 group). Feed particle were 0.5 - 1 mm in size. Sturgeons were fed three times a day the experimental diets (3% body weight daily). At the beginning (t_0) and at the end of the feeding trial (t_1), after a 10-hour fasting period, the required fish were sampled, euthanized with a lethal dose of MS222 (0.3 g/L; Merck KGaA, Darmstadt, Germany) and properly stored for further analyses.

Biometry

For growth measurements, 20 fish per tank (60 fish per dietary group) at both t_0 and t_1 were randomly collected from the different tanks. Wet weight was measured with an analytical balance (precision 0.1 mg). The specific growth rate (SGR) was calculated as follows: $SGR\% = [(\ln W_f - \ln W_i) / t) \times 100$, where W_f is the wet weight determined at t_1, W_i the wet weight determined at t0, and t the number of days (60). During the trial, dead fish were removed and recorded to estimate the final survival rate.

Fatty acids composition.

Lipid content and fatty acids composition of experimental diets (3 samples per diet) and fish fillet (3 fish per tank; 9 fish per dietary group) were determined after sample homogenization (homogenizer MZ 4110, DCG, Eltronic, Monza, Italy) and freeze-drying (Edwards EF4, Crawley, Sussex, England). Lipid extraction was carried out on lyophilized powders with the Folch's method [**46**] for diets and with Microwave-Assisted Extraction method for fish [**47**]. All lipid extracts were evaporated under laminar flow inert gas (N_2) until constant weight to determine lipid content; then, they were re-suspended in 0.5 mL of n-epthane for fatty acids analysis. Fatty acids methyl esters (FAME) were prepared according to Canonico et al. [**48**] using methyl ester of nonadecanoic acid (19:0; Dr. Ehrenstorfer GmbH, Augsburg, Germany) as internal standard. A gas-chromatographic (GC) system (Agilent-6890, Milano, Italy) coupled with a Mass Selective Detector (MS) (Agilent-5973N quadrupole, Milano, Italy) was used to determine FAME. A CPS ANALITICA CC-wax-MS (30 m × 0.25 mm ID, 0.25 μm film thickness) capillary column was used to separate FAME. Instrumental conditions were set up according to Truzzi et al. [**49**]. Analyses were carried out on three aliquots per sample. For each aliquot, at least three runs were performed on the GC-MS.

Histology

Liver, small intestine and spiral valve from 5 different fish per tank (15 per dietary group) were randomly collected at both t_0 and t_1. Samples were fixed by immersion in Bouin's solution for 24 hours, washed three times with 70% ethanol for 15 minutes and finally preserved in a new 70% ethanol solution. After a dehydration through graded ethanol solutions (80, 95 and 100 %), samples were washed with xylene (Bio-Optica, Milano, Italy) and embedded in paraffin (Bio-Optica). Solidified paraffin blocks were cut with a microtome (Leica RM2125 RTS, Nussloch, Germany) and 5 μm sections were stained with Mayer haematoxylin and eosin Y (Merck KGaA). Sections were observed using a Zeiss Axio Imager.A2 (Oberkochen, Germany) microscope to study the hepatic parenchyma and the morphology of small intestine, pyloric caecum and spiral valve. Images were acquired by mean of a combined colour digital camera Axiocam 503 (Zeiss). In order to evaluate the percentage of fat fraction in the liver (PFF), three sections per fish (15 fish per dietary group; n = 3), at 100 μm intervals, were acquired and analysed by mean of the ImageJ software setting a homogeneous threshold value according to Zarantoniello et al. [**38**] (chapter 3.1). Non evaluable areas on sections, such as blood vessels and bile ducts, were not considered. Results were reported as mean ± SD of the area occupied by fat on the total hepatic parenchyma analysed on the section. A semi-quantitative evaluation was performed on small intestine, pyloric caecum and spiral valve morphology based on mucosal folds height, enterocytes supranuclear vacuolization and abundance

of goblet cells as previously described in Urán et al. [**66**]. Specifically, for the morphometric evaluation of mucosal folds height, ten transversal sections per fish (15 fish per dietary group) of small intestine, pyloric caecum and spiral valve, at 200 μm intervals, were analysed as described in Cardinaletti et al. [**28**]. All the undamaged and non-oblique folds were measured (at least 150 measurements per fish) using ZEN 2.3 software (Zeiss) and measurements were reported as height mean ± SD (μm) [**28**]. For the semi-quantitative analysis of supranuclear vacuoles and goblet cells, 3 whole intestine circular transversal sections per fish (15 fish per dietary group), at 200 μm intervals, were analysed. The sections were analysed by experienced staff in two independent blinded evaluations and an arbitrary unit was assigned as described in Panettieri et al. [**51**]. Scores were assigned as follows: supranuclear vacuoles + = scattered, + + = abundant; goblet cells + = 0/4 per villus, + + > 4 per villus.

FTIR measurements

Samples of liver and small intestine collected at t_1 from 2 different fish per tank (6 fish per dietary group), were quickly dissected and immediately frozen at -80 °C. Samples were then prepared for infrared spectroscopy (IR) measurements according to Notarstefano et al. [**52**]. Particularly, from the middle part of each liver and small intestine samples, three thin sections (10 μm thick) were cut at 200 μm intervals by using a chryomicrotome (Microm HM 505 N, Neuss, Germany) and deposited onto CaF_2 optical windows (1 mm thick, 13 mm diameter) for FTIRM analysis [**52**]. IR measurements were performed at 20 °C by using a Bruker Invenio FTIR spectrometer equipped with an FPA detector operating at liquid nitrogen temperature (Bruker Optics, Ettlingen, Germany). On each section, two areas were selected on which IR maps were collected in transmission mode in the spectral range 4000-800 cm^{-1} (spectral resolution 4 cm^{-1}; spatial resolution 2.56 × 2.56 μm^2). IR maps with different size were collected on liver and small intestine sections due to the different morphology and structure of the samples (164 × 164 μm^2 size, 4096 pixel/spectra, 128 scans on liver sections, and 328 × 164 μm^2, 8192 pixel/spectra, 256 scans on small intestine sections). All raw IR maps were corrected for the contributions of carbon dioxide and water vapor, and then vector normalized to avoid artifacts due to local thickness variations (OPUS 7.0 software, Bruker Optics, Ettlingen, Germany).

Liver samples. False color images representing the topographical distribution of total lipids, fatty acids, unsaturated alkyl chains, proteins and glycogen were built from each IR map. For this purpose, the following spectral Regions of Interest (ROI) were investigated: 2999-2829 cm^{-1} (total lipids, arbitrary color scale 0-10; LIP); 1775-1724 cm^{-1} (fatty acids, arbitrary color scale 0-3; FA); 3035-2999 cm^{-1} (unsaturated alkyl groups, arbitrary color scale 0-0.5; CH); 1718-1486 cm^{-1} (total proteins, arbitrary color scale 0-10; PRT), and 1073-980 cm^{-1} (glycogen, arbitrary color scale 0-3; GLY). Due

to the different absorption of the infrared radiation (molar extinction coefficient, ε) showed by the analyzed macromolecular components, different color scales were adopted; in any case, dark blue color represented the minimum of the infrared absorption, while white/light pink color the maximum one. On all the IR spectra of each map, the integrated areas of the above defined ROI were calculated, and the values used for the following band area ratios: LIP/TBM, FA/TBM, CH/TBM, PRT/TBM, and GLY/TBM. TBM is representative of the total tissue biomass and was calculated by the sum of the integrated areas 2999-2828 and 1801-945 cm^{-1}.

Small intestine samples. False color images representing the topographical distribution of total lipids, proteins and carbohydrates were built from each IR map. For this purpose, the following ROI were investigated: 2999-2828 cm^{-1} (total lipids, arbitrary color scale 0-3; LIP); 1718-1486 cm^{-1} (total proteins, arbitrary color scale 0-10; PRT) and 1135-1006 cm^{-1} (total carbohydrates, arbitrary color scale 0-2; CARBO). Due to the different absorption of the infrared radiation (molar extinction coefficient, ε) showed by the analyzed macromolecular components, different color scales were adopted; in any case, dark blue color represented the minimum of the absorption, while white/light pink color the maximum one. From each IR map, ca. 300 IR spectra, representative of the small intestine epithelial absorption portion, were extracted and integrated under the above defined ROI. These values were used for the following band area ratios: LIP/TBM, PRT/TBM, and CARBO/TBM. TBM is representative of the total tissue biomass and was calculated by the sum of the integrated areas 2999-2828 and 1801-945 cm^{-1}.

Sturgeon gut microbiome

RNA extraction and cDNA synthesis. Gut samples from 3 different fish per tank (9 fish per dietary group) were collected at t_0 and t_1 and processed as previously described by Zarantoniello et al. [**38**] (chapter 3.1). The obtained cell pellets were covered with RNA later Stabilization Solution (Ambion, Foster City, CA, USA) and stored at -80 °C until the extraction of total microbial RNA performed by Quick-RNA Miniprep kit (Zymo Research, CA, USA). The quantity and purity of the extracted RNA were checked using a Nanodrop ND 1000 (Thermo Fisher Scientific, Waltham, Massachusetts, USA). Moreover, the absence of residual DNA contamination was checked by PCR as described by Garofalo et al. [**53**]. Each sample RNA (10 µL) was reverse-transcribed in cDNA using oligo (dT) and random hexamer primers from SensiFAST cDNA Synthesis Kit for RT-qPCR (Bioline, London, UK).

16S rRNA gene amplicon target sequencing. The portion of 16S rRNA gene (V3-V4 region) from each cDNA sample was amplified by PCR as previously described by Klindworth et al. [**54**]. The PCR products were further processed and sequenced by MiSeq Illumina instrument (Illumina, San Diego, California, USA) following the procedure detailed by Osimani et al. [**18**].

Molecular analyses

RNA extraction and cDNA synthesis. Total RNA extractions from liver and small intestine samples from 5 fish per tank (15 fish per dietary group) at both t_0 and t_1 were performed using RNAzol RT reagent (R4533, Merck KGaA). Total RNA extracted was eluted in 40 µl of RNase-free water (Qiagen) and stored at -80°C until use. The final RNA concentration was determined by a NanoPhotometer P-Class (Implen, München, Germany) and the RNA integrity was verified by GelRed™ staining of 28S and 18S ribosomal RNA bands on 1% agarose gel. The cDNA synthesis was performed with LunaScript RT SuperMix Kit (New England Biolabs, Ipswich, Massachusetts, USA) using 1 µg of total RNA.

Real-Time PCR. PCRs were performed in an iQ5 iCycler thermal cycler (Bio-Rad, Hercules, California, USA). Reactions were set on a 96-well plate by mixing, for each sample, 1µL cDNA diluted 1:10, 5 µL of 2× concentrated iQ™ Sybr Green (Bio-Rad, Hercules, California, USA) as fluorescent intercalating agent, 0.3 µM of forward primer and 0.3 µM of reverse primer. The thermal profile for all reactions was 3 min at 95 °C and then 45 cycles of 20 s at 95 °C, at primers specific annealing temperature (for details, please see Table 2), and 20 s at 72 °C. At the end of each cycle florescence was monitored and the melting curve analyses showed in all cases one single peak. Relative quantification of the expression of genes involved in fish growth (insulin-like growth factor 1, *igf1*), stress (heat shock cognate 70-kd protein, tandem duplicate 1, *hsp70.1*) and immune response (tumor necrosis factor a, *tnfa*) was performed. Actin, beta 1 (*actb1*) and glyceraldehyde-3-phosphate dehydrogenase (*gapdh*) were used as internal standards in each sample to standardize the results by eliminating variation in mRNA and cDNA quantity and quality. No amplification products were observed in negative controls and no primer-dimer formations were observed in the control templates. The data obtained were analysed using the iQ5 optical system software version 2.0 (Bio-Rad), including GeneEx Macro iQ5 Conversion and Genex Macro iQ5 files. Primer sequences used in this study are reported in Table 2.

Table 2. Sequences and annealing temperature (A.T.) of the primers used for the present study.

Gene	Forward Primer (5'-3')	Reverse Primer (5'-3')	A. T. (°C)
igf1	AGCTGAGCTTGTGGACAC	AAGCAGCACTCATTCACGAT	53
hsp70.1	ACAGCCATGTTGTATACTGAGTCC	TGCACACCTTCTCCAGTTCTT	58
tnfa	TGTGTCTGTAGAGCACTCCGAT	CATGGCCAGCAAGTCGAT	53
actb1	GTTGGTATGGGACAGAAGGACA	CCAGTTGGTAACAATGCCGT	60
gapdh	CATTTGATGTTGGCTGGGT	CTTTCTGGGAAGGTGGAGGT	59

Statistical analyses

All data (except for microbiome) were analysed by t-test, with diet as the explanatory variable and presented as mean ± SD. The statistical software package Prism5 (GraphPad Software) was used. Significant differences between Hi0 and Hi50 were indicated as follows: ns, non-significant; * $p < 0.05$; ** $p < 0.01$; *** $p < 0.001$ and **** $p < 0.0001$. For microbiome bioinformatics analyses, raw reads were first merged with the FLASH software and analysed with the QIIME 1.9.0 software [**55**]; the detailed pipeline was described by Ferrocino et al. [**56**]. OTUs clustering was obtained at 97% of similarity and centroids sequencing were mapped against the Greengenes 16S rRNA gene database. OTU tables generated by QIIME were rarefied at the lowest number of reads and showed the highest reached taxonomic resolution. The vegan package of R was used for the alpha diversity calculation.

Results

Water chemistry

Nitrite (NO_2^-), nitrate (NO_3^-) and phosphate (PO_4^{3-}) weekly trends are shown in Figure 1. No significant differences were detected between the two experimental groups. Ammonia values were lower than 0.05 mg/L for both Hi0 and Hi50 at each sampling time.

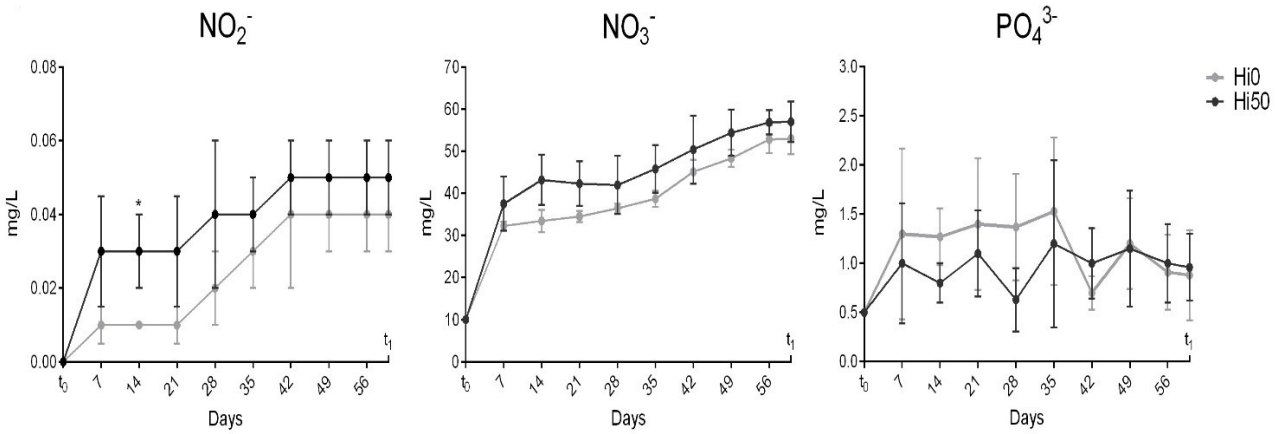


Figure 1. Trend of aquaponic system water parameters measured during the trial. Values are shown as mean (dots) ± SD (n = 3).

Sturgeon survival and growth

Siberian sturgeon survival was significantly ($p < 0.0001$) lower in Hi50 (80 ± 4 %) compared to Hi0 (97 ± 3 %). Considering the specific growth rate (% weight gain day^{-1}), Hi50 (1.8 ± 0.9 %) was significantly ($p < 0.0001$) lower than Hi0 (2.9 ± 0.8 %).

Fatty acids composition

Diets. Figure 2a shows the percentages of fatty acids classes of the two experimental diets. The Hi50 diet resulted in a significantly ($p < 0.0001$) higher percentage of SFA (44.2 ± 1.0 %) and significantly lower MUFA (8.6 ± 0.3 %; $p < 0.0001$) and PUFA (47.3 ± 1.2 %; $p < 0.01$) compared to Hi0 diet (26.1 ± 0.3, 22.7 ± 0.1 and 51.2 ± 0.5 % for SFA, MUFA and PUFA, respectively). In addition, the inclusion of BSF full-fat prepupae meal in the diet resulted in a significant ($p < 0.0001$) decrease of n3 (40.1 ± 0.5 and 19.9 ± 1.0 % for Hi0 and Hi50, respectively) and n9 (13.7 ± 0.1 and 8.6 ± 0.3 % for Hi0 and Hi50, respectively) percentages and a significant ($p < 0.0001$) increase in n6 percentage (10.9 ± 0.1 and 27.4 ± 0.7 % for Hi0 and Hi50, respectively). Therefore, the n6/n3 ratio showed significant differences ($p < 0.0001$) between experimental diets (0.27 ± 0.05 and 1.40 ± 0.10 for Hi0 and Hi50, respectively; Fig. 2b). Considering the specific fatty acids composition (Table 3), the Hi50 diet was characterized by a significantly higher percentages of lauric (12:0), stearic (18:0), 7-hexadecenoic (16:1n9), linoleic (18:2n6) and α-linolenic (18:3n3) acids compared to the Hi0 diet. In the Hi0 diet palmitoleic (16:1n7), oleic (18:1n9), eicosapentaenoic (EPA, 20:5n3) and docosahexaenoic (DHA, 22:6n3) acids were significantly more abundant. Finally, the DHA/EPA ratio was significantly ($p < 0.05$) higher in the Hi50 diet compared to the Hi0 diet.

Sturgeon. As shown in Figure 2c,d, no significant differences were observed between the experimental groups at t_0 in terms of either fatty acids composition (SFA: 21.5 ± 0.4 and 20.9 ± 0.4 %; MUFA: 30.0 ± 0.1 and 29.8 ± 0.2 %; PUFA: 48.5 ± 0.5 and 49.3 ± 0.5 %; n3: 32.0 ± 0.5 and 32.8 ± 0.5 %; n6: 16.2 ± 0.1 and 16.2 ± 0.1 %; n9: 22.6 ± 0.1 and 22.5 ± 0.1 % for Hi0 and Hi50, respectively) or n6/n3 ratio (0.51 ± 0.03 and 0.49 ± 0.01 for Hi0 and Hi50, respectively). In terms of the specific composition, slightly significant differences were detected for some fatty acids due to physiological differences among fish (for specific details, see Table 3).

Considering fatty acids content of Siberian sturgeon fillets at t_1 (Fig. 2e), no significant differences were detected between the experimental groups in terms of SFA (22.9 ± 0.3 and 23.0 ± 0.5 % for Hi0 and Hi50 respectively), MUFA (29.0 ± 0.1 and 29.0 ± 0.6 % for Hi0 and Hi50, respectively), PUFA (48.2 ± 0.5 and 48.1 ± 1.2 % for Hi0 and Hi50, respectively) and n9 (19.9 ± 0.1 and 19.9 ± 0.5 % for Hi0 and Hi50, respectively) content. However, the Hi50 group was characterized by a significantly ($p < 0.0001$) lower n3 percentage (24.9 ± 1.2 %) and a significantly ($p < 0.0001$) higher n6 percentage (23.0 ± 0.5 %) than Hi0 (32.6 ± 0.5 and 15.4 ± 0.1 % for n3 and n6, respectively).

Consequently, the n6/n3 ratio (Fig. 2f) was significantly ($p < 0.0001$) higher in Hi50 (0.93 ± 0.05) compared to Hi0 (0.47 ± 0.07).

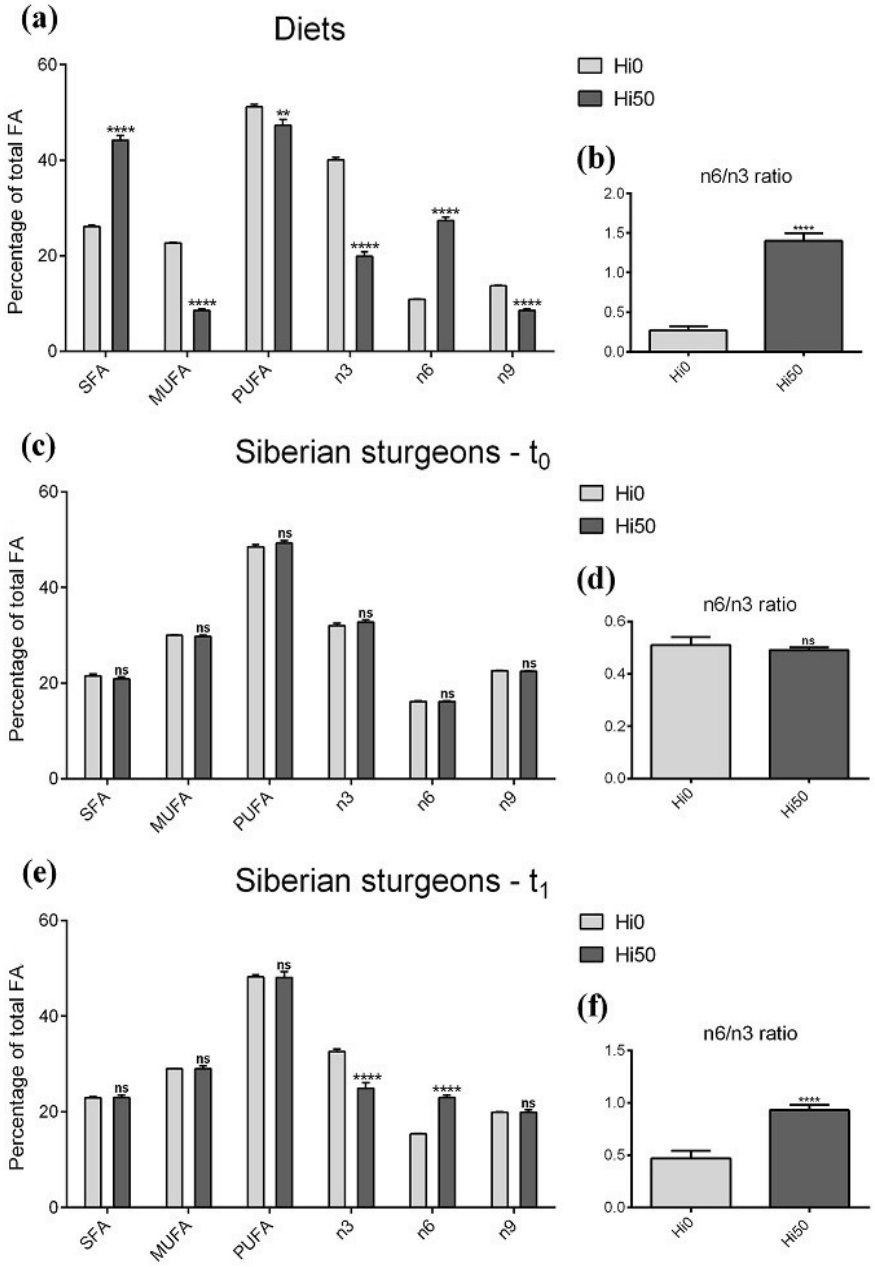


Figure 2. Percentage of SFA, MUFA and PUFA (as % of total fatty acids) and omega 3 (n3), omega 6 (n6) and omega 9 (n9) fatty acids contribution (%) to lipid profile. (**a**,**b**) Experimental diets including 0 and 50 % of BSF prepupae meal respect to FM (Hi0 and Hi50); (**c**,**d**) Siberian sturgeon fillets at t_0; (**e**,**f**) Fillets of Siberian sturgeon fed the different diets at t_1. Significant differences between Hi0 and Hi50, compared within the same fatty acids class, are indicated as follows: *ns* non-significant; * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$ and **** $p < 0.0001$. Values are shown as mean ± SD (n = 3 for experimental diets; n = 9 for sturgeon at both t_0 and t_1).

In terms of specific fatty acids composition at t_1 (Table 3), Hi50 showed significantly higher percentages of lauric (12:0), stearic (18:0), oleic (18:1n9), linoleic (18:2n6) and dihomo-γ-linolenic (20:3n6) acids than Hi0. Conversely, significantly higher percentages of α-linolenic (18:3n3), eicosapentaenoic (20:5n3) and docosahexaenoic (22:6n3) acids were detected in Hi0 respect to Hi50. The DHA/EPA ratio was significantly ($p < 0.0001$) higher in Hi50 compared to Hi0.

Table 3. Fatty acids composition (as % of total fatty acids) of experimental diets and Siberian sturgeon at t_0 and t_1.

	Diets		Sturgeon			
			t_0		t_1	
	Hi0	**Hi50**	**Hi0**	**Hi50**	**Hi0**	**Hi50**
10:0	0.020±0.001	0.570±0.010****	0.005±0.001	0.004±0.001	0.003±0.001	0.004±0.001
12:0	0.18±0.01	8.1±0.7****	0.15±0.04	0.12±0.03	0.10±0.03	2.35±0.60****
13:0	0.06±0.001	n.d.	0.032±0.001	0.029±0.001	0.030±0.002	0.044±0.005
14:0	5.67±0.06	4.6±0.3**	2.80±0.09	2.67±0.04**	3.81±0.12	3.73±0.21^{ns}
14:1n5	0.53±0.01	n.d.	0.34±0.01	0.32±0.01	0.41±0.01	0.82±0.07****
15:0	0.61±0.01	0.58±0.03^{ns}	0.61±0.01	0.59±0.01***	0.60±0.01	0.57±0.01****
15:1n5	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.
16:0	14.5±0.3	17.1±0.6**	13.9±0.1	13.7±0.1***	14.7±0.2	12.3±0.1****
16:1n9	0.36±0.01	6.3±0.3****	0.49±0.01	0.48±0.01	0.49±0.01	0.74±0.06**
16:1n7	6.25±0.04	n.d.	4.37±0.01	4.29±0.01****	5.81±0.11	5.60±0.42^{ns}
16:2n7	0.28±0.01	n.d.	0.30±0.02	0.28±0.01	0.13±0.02	0.21±0.01
17:0	0.53±0.09	n.d.	0.48±0.09	0.55±0.08	0.50±0.01	0.41±0.06
17:1n7	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.
18:0	3.87±0.01	13.1±1.8***	3.21±0.02	3.00±0.02****	2.95±0.39	3.44±0.16**
18:1n9	9.94±0.06	2.30±0.20****	17.7±0.1	17.6±0.1*	16.5±0.3	17.1±0.2***
18:1n7	2.26±0.01	n.d.	2.66±0.01	2.66±0.02^{ns}	2.91±0.04	2.61±0.17****
18:2n6	9.06±0.03	27.4±2.3***	12.9±0.1	12.9±0.1^{ns}	12.5±0.3	17.5±1.3****
18:3n6	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.
18:3n3	1.91±0.06	3.4±0.3**	2.51±0.01	2.51±0.01^{ns}	2.03±0.12	1.86±0.06**
20:0	0.36±0.01	n.d.	0.24±0.01	0.23±0.01	0.19±0.01	0.12±0.02
20:1n9	1.83±0.03	n.d.	3.17±0.04	3.19±0.01^{ns}	2.25±0.12	1.80±0.27***
20:2n6	0.33±0.03	n.d.	1.26±0.05	1.24±0.03^{ns}	1.10±0.02	1.93±0.07****
20:3n6	0.26±0.02	n.d.	0.57±0.08	0.53±0.02^{ns}	0.53±0.01	1.26±0.07****
21:0	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.
20:4n6	1.23±0.01	n.d.	1.48±0.02	1.49±0.02^{ns}	1.27±0.04	2.27±0.16****
20:3n3	0.17±0.01	n.d.	0.43±0.01	0.43±0.01	0.30±0.02	0.30±0.04
20:5n3	17.3±0.2	4.8±0.4****	6.15±0.04	6.26±0.03****	9.29±0.18	4.51±0.05****
22:0	0.24±0.01	n.d.	0.081±0.001	0.077±0.003	0.063±0.003	0.039±0.009
22:1n9	1.53±0.03	n.d.	1.19±0.05	1.19±0.03^{ns}	0.62±0.06	0.25±0.14****
23:0	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.
24:0	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.
22:6n3	20.7±0.3	11.7±1.5***	22.9±0.1	23.6±0.2****	21.0±0.3	18.2±1.7***
24:1n9	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.
DHA/EPA	1.19±0.03	2.5±0.5*	3.7±0.1	3.8±0.1*	2.26±0.06	4.0±0.3****

Significant differences between Hi0 and Hi50 within rows and separately from diets, t_0 and t_1 are indicated as follows: ns, non-significant; * $p<0.05$; ** $p<0.01$; *** $p<0.001$ and **** $p<0.0001$. Values are shown as mean ± SD (n = 3 for experimental diets; n = 9 for sturgeon at both t_0 and t_1). Statistical analysis was performed only for fatty acids > 0.5% (fatty acids with a percentage < 0.5% were excluded from any statistical analyses because their concentrations were close to the limit of detection).

Histology

Histological analyses at t_0 were performed in order to evaluate liver and small intestine histological integrity at the beginning of the experiment. Sturgeons exhibited a homogeneous hepatic parenchyma with hepatocytes characterized by a moderate degree of intra-cytoplasmic lipid deposition (Fig. 3a,b). The percentage of fat fraction (PFF) in the liver parenchyma did not show significant differences between Hi0 (49.2 ± 7.6 %) and Hi50 (51.5 ± 9.4 %) groups at t0. Histology of the small intestine (Fig. 3c,d) evidenced a regular morphology of mucosal folds, with finger-shaped folds formed by a mono-stratified epithelial layer of enterocytes intercalated with goblet cells, followed by a thin submucosal layer surrounded by the outer muscular layer.

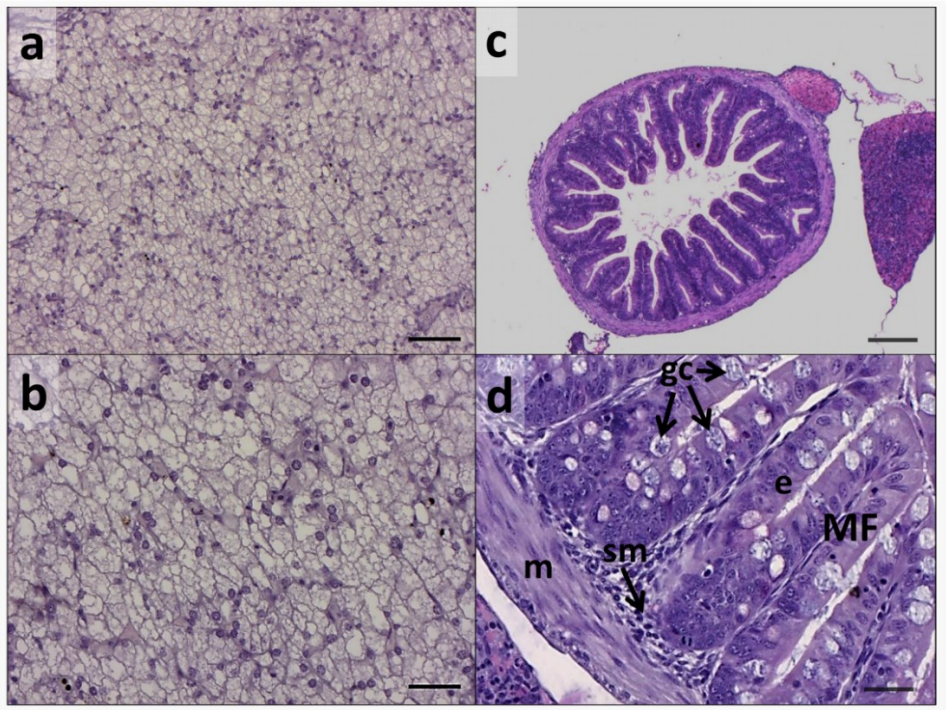


Figure 3. Example of histomorphology of Siberian sturgeon liver and intestine at t_0. (**a**,**b**) liver; (**c**,**d**) intestine. Scale bars: (**a**) 100 µm; (**b**,**d**) 50 µm; (**c**) 500 µm. Letters: m = muscular layer; sm = submucosal layer; gc = goblet cells; e = enterocytes; MF = mucosal folds.

Liver analysis at t_1 showed a considerable difference in parenchyma lipid accumulation between Hi0 and Hi50. Specifically, swollen hepatocytes filled of fat with limited cytoplasm were observed in livers of Hi0 sturgeons (Fig. 4a,b), while fat deposition was significantly reduced in the Hi50 group (Fig. 4c,d). PFF analysis at t_1 confirmed this result highlighting significant differences in lipid deposition ($p < 0.001$) between Hi0 (55.3 ± 2.7 %) and Hi50 (20.9 ± 7.0 %) (Fig. 4e).

Representative histological images of small intestine (SI), pyloric caecum (PC) and spiral valve (SV) are shown in Figure 5. In the morphometric analysis of these gut tracts (Fig. 5m), no significant differences were detected between the groups at t_0, while mucosal folds atrophy, with a significant reduction of folds length, was observed at t_1 in Hi50 SI (Fig. 5c,d; $p < 0.0001$), PC (Fig. 5g,h, $p <$

0.0001) and SV (Fig. 5k,l, $p < 0.05$) compared to the Hi0 group (Fig. 5a,b,e,f,i,j for SI, PC and SV, respectively). In addition, a significant ($p < 0.0001$) reduction of supranuclear vacuoles in SI and PC and a significant ($p < 0.01$) reduction in the relative abundance of goblet cells in SI and SV were observed in Hi50 compared to Hi0.

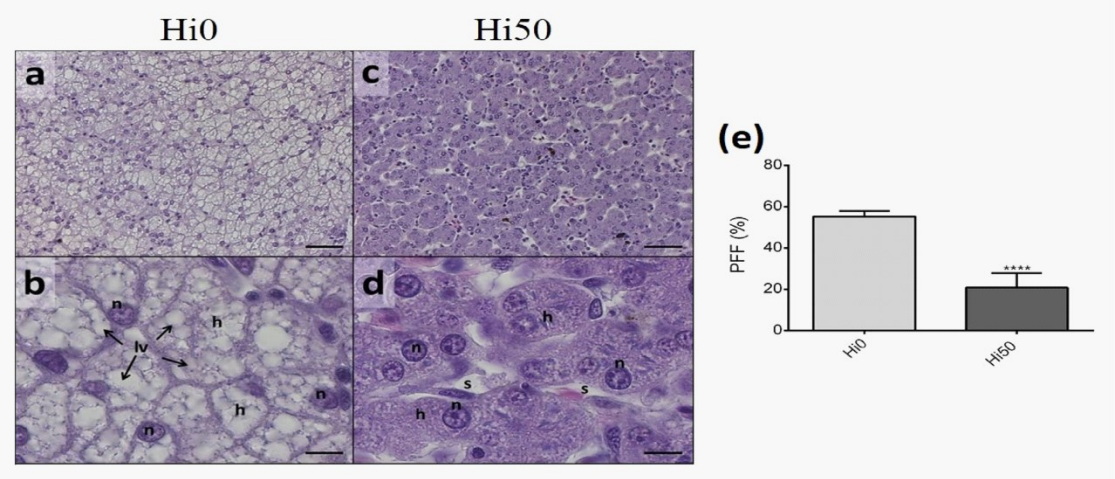
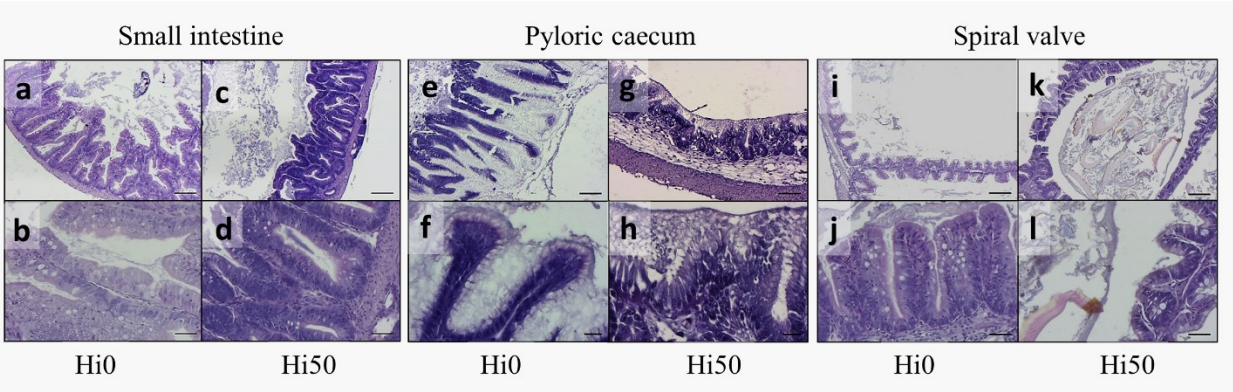


Figure 4. Example of liver histomorphology and percentage of fat fraction (PFF) in hepatic tissue of Siberian sturgeon at t_1. (**a**,**b**) Hi0; (**c**,**d**) Hi50; (**e**) PFF. Histology scale bars: (**a**,**c**) 50 µm; (**b**,**d**) 10 µm. Letters: h = hepatocyte, n = nucleus, lv = lipid vesicles, s = hepatic sinusoids. For PFF, values are shown as percentage mean ± SD (n = 15). Significant differences between Hi0 and Hi50 are indicated as follows: ns, non-significant; * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$ and **** $p < 0.0001$. Sturgeon fed diets including 0 and 50 % of BSF prepupae meal (Hi0 and Hi50, respectively).



(m)

		Mucosal folds length (µm)			Supranuclear vacuoles			Goblet cells		
		SI	PC	SV	SI	PC	SV	SI	PC	SV
t_0	Hi0	394.2±19.3	587.6±36.2	159.3±19.7	++	++	+	++	+	++
	Hi50	387.1±16.9	601.8±24.7	152.1±13.2	++	++	+	++	+	++
t_1	Hi0	410.3±10.5	600.9±21.74	162.3±16.36	++	++	+	++	+	++
	Hi50	231.5±11.28****	312.5±16.08****	150.3±12.21*	+***	+***	+	+**	+	+**

Figure 5. Siberian sturgeon small intestine (SI), pyloric caeca (PC) and spiral valve (SV) histology at t_1 and histological indexes (mucosal folds length, supranuclear vacuoles abundance and goblet cells number) calculated in these gut tracts. (**a**-**d**) small intestine; (**e**-**h**) pyloric caecum; (**i**-**l**) spiral valve. Histology scale bars: (**a**,**c**,**e**,**g**) 100 µm; (**b**,**d**,**f**,**h**,**j**,**l**) 50 µm; (i,k) 200 µm. For histological indexes (**m**), values are showed as mean ± SD (n = 15). Scores: supranuclear vacuoles + scattered, ++ abundant; goblet cells + 0 to 4 per villus, ++ more than 4 per villus. Significant differences, calculated within the same sampling time, between Hi0 and Hi50 are indicated as follows: ns, non-significant; *$p < 0.05$, **$p < 0.01$, ***$p < 0.001$ and ****$p < 0.0001$. Sturgeon fed diets including 0 and 50 % of BSF prepupae meal (Hi0 and Hi50, respectively).

FTIR analysis

The spectral analysis of liver samples collected from Siberian sturgeons at t_1 showed differences in the biochemical composition between Hi0 and Hi50.

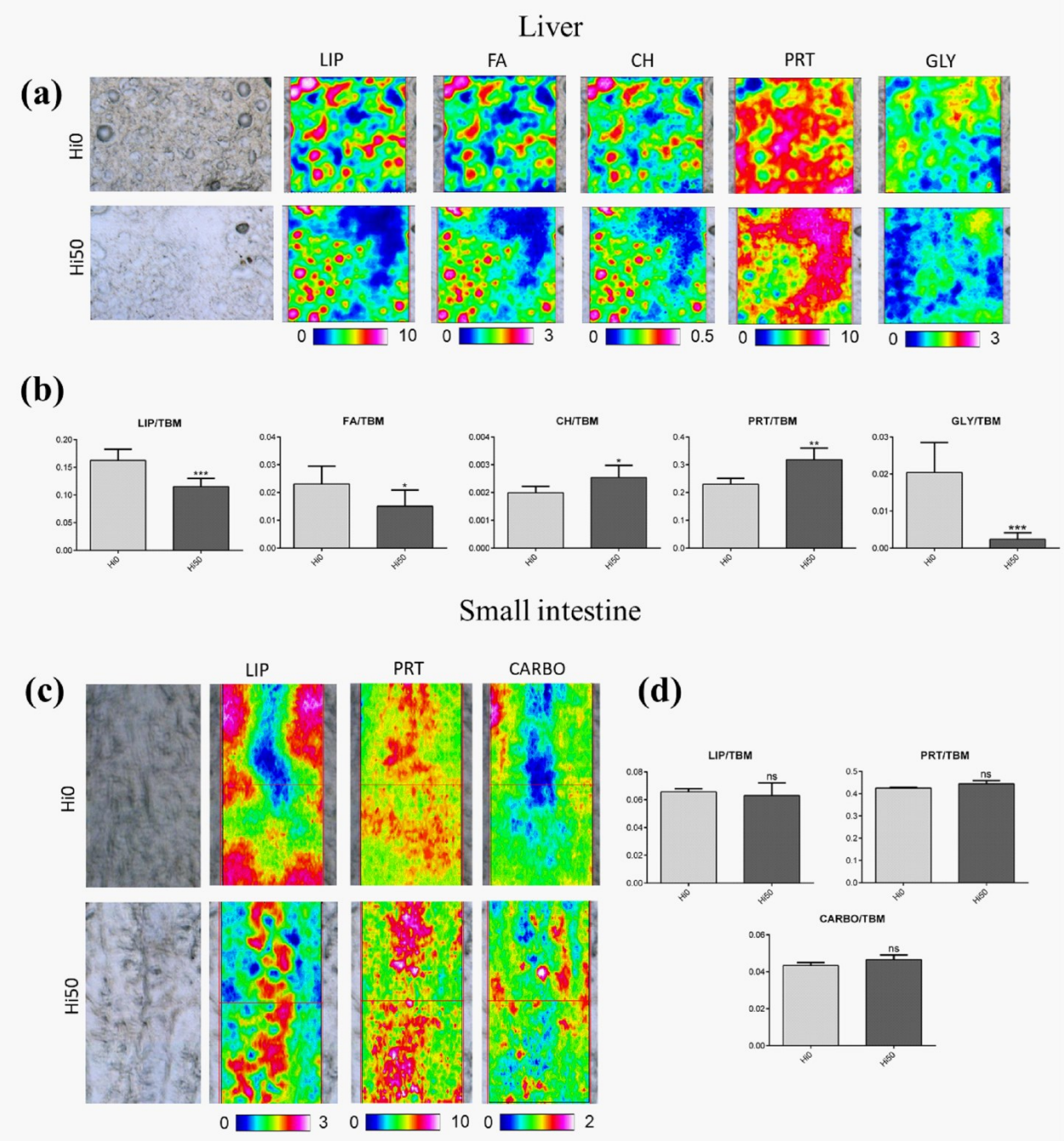


Figure 6. Spectral analysis of liver and small intestine samples of Siberian sturgeon collected at t_1. (**a**) Microphotographs of liver samples and topographical distribution of: lipids (LIP), fatty acids (FA), unsaturated alkyl chains (CH), proteins (PRT maps), and glycogen (GLY maps) (map size 164 × 164 μm^2). (**b**) Statistical analysis of liver biochemical composition: LIP/TBM (relative amount of total lipids), FA/TBM (relative amount of fatty acids), CH/TBM (degree of unsaturation in lipid alkyl chains), PRT/TBM (relative amount of total proteins) and GLY/TBM (relative amounts of glycogen). (**c**) Microphotographs of small intestine samples and topographical distribution of: lipids (LIP maps), proteins (PRT maps) and carbohydrates (CARBO maps) (map size 328 × 164 μm^2). (**d**) Statistical analysis of small intestine biochemical composition: LIP/TBM (relative amount of total lipids), PRT/TBM (relative amount of total proteins) and CARBO/TBM (relative amount of carbohydrates). Significant differences between Hi0 and Hi50 are indicated as follows: *ns* non-significant; *p < 0.05, **p < 0.01, ***p < 0.001 and ****p < 0.0001. Data are reported as mean ± SD (n = 6). Sturgeons fed diets including 0 and 50 of BSF meal (Hi0 and Hi50, respectively).

The IR maps (Fig. 6a), as well as the statistical analysis of specific band area ratios (Fig. 6b), showed a significant decrease of total lipids (LIP maps; LIP/TBM, $p < 0.001$), fatty acids (FA maps; FA/TBM, $p < 0.05$) and glycogen (GLY maps; GLY/TBM, $p < 0.01$) in Hi50 compared to Hi0. An increase in unsaturated lipids (CH maps; CH/TBM, $p < 0.05$) and proteins (PRT maps; PRT/ TBM, $p < 0.01$) in Hi50 was also observed. For the small intestine samples, both the IR maps (LIP, PRT and CARBO maps; Fig. 6c) and the statistical analysis of specific band area ratios (LIP/TBM, PRT/TBM and CARBO/TBM; Fig. 6d) did not show significant modifications between Hi0 and Hi50.

Sturgeon gut microbiome

No significant differences were observed in the alpha diversity values (Shannon, Chao1 and number of OTUs) between Hi0 and Hi50 at both t_0 and t_1. However, a higher number of bacterial groups at genus or family level were identified in Hi50 (22 groups) compared to Hi0 (17 groups) samples at t_1. Relative abundances of bacterial taxa were examined to determine the effect of the diet on gut microbiota composition. The average values (Fig. 7) of the biological replicates at both sampling times were found to be very similar.

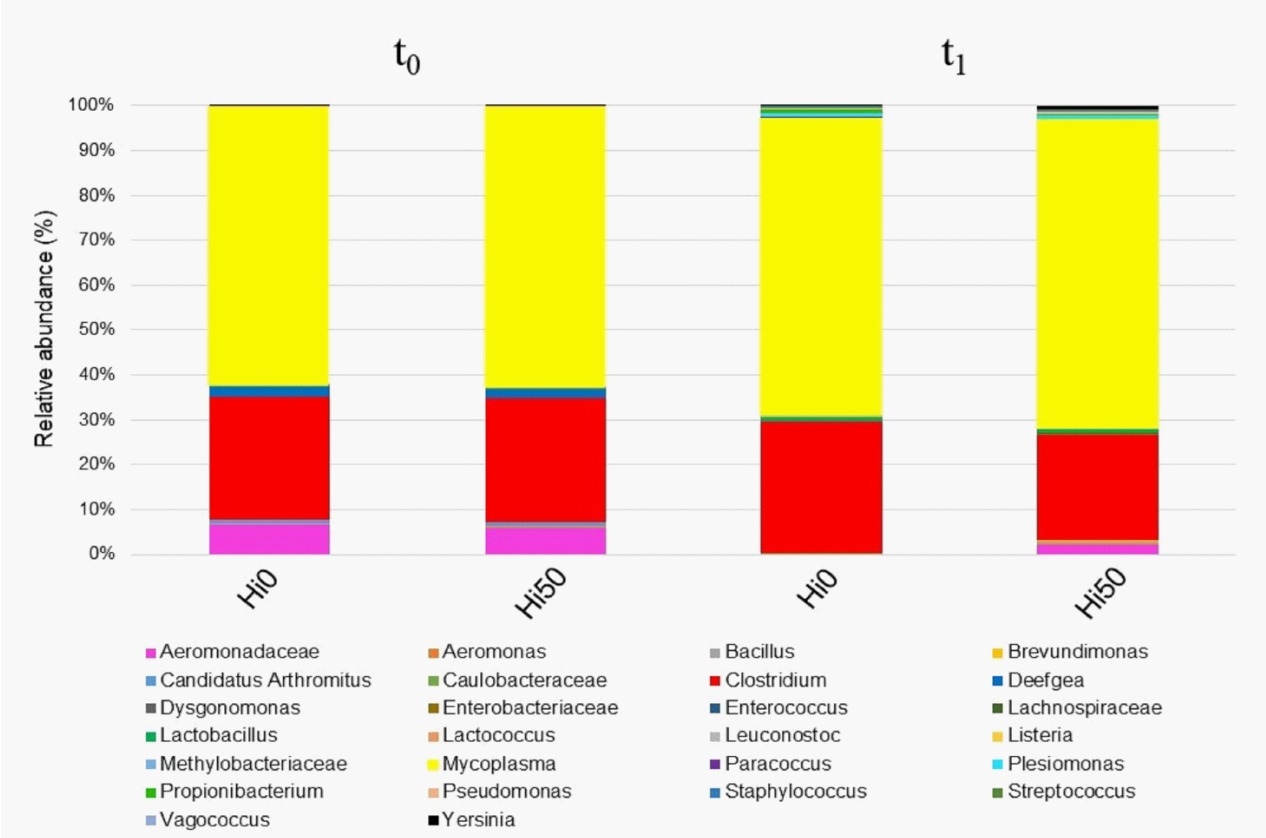


Figure 7. Relative abundances (%) of bacterial community in Siberian sturgeon gut samples at both t_0 and t_1 as identified by MiSeq Illumina. Sturgeon fed diets including 0 and 50 % of BSF prepupae meal (Hi0 and Hi50, respectively).

The taxonomic analysis showed the dominance (> 58%) of *Mycoplasma* in all samples analysed, followed by *Clostridium*, with relative abundances comprised between 22.6% (Hi50, t_1) and 28.3% (Hi0, t_1). Aeromonadacean bacteria were found in gut samples from both Hi0 and Hi50 at t_0 (about 6%), and exclusively in Hi50 sampled at t_1 with the relative abundance of 2.1%. Bacteria of the genus *Deefgea* were present in both experimental groups exclusively at t_0 with relative abundance of about 2%. Additional bacteria were detected sporadically in some samples, with a relative abundance < 1%. *Lactobacillus*, *Paracoccus*, *Propionibacterium* and *Streptococcus* were identified solely at t_1 in both experimental groups, while *Listeria* was found only in Hi50.

Real-time PCR results

Real-time PCR analyses were performed on liver samples in order to test the expression of genes involved in fish growth (*igf1*) and stress response (*hsp70.1*). Gene expression of *tnfa* was investigated in intestine samples. As shown in Figure 8, the expression of the genes analysed did not show significant differences between the experimental groups at t_0. At t_1, results evidenced a significant ($p < 0.01$) downregulation for *igf1* (Fig. 8a) and a significant ($p > 0.01$) upregulation for *hsp70.1* (Fig. 8b) in Hi50 compared to Hi0. For *tnfa* (Fig. 8c), no significant differences in gene expression were detected between the experimental groups.

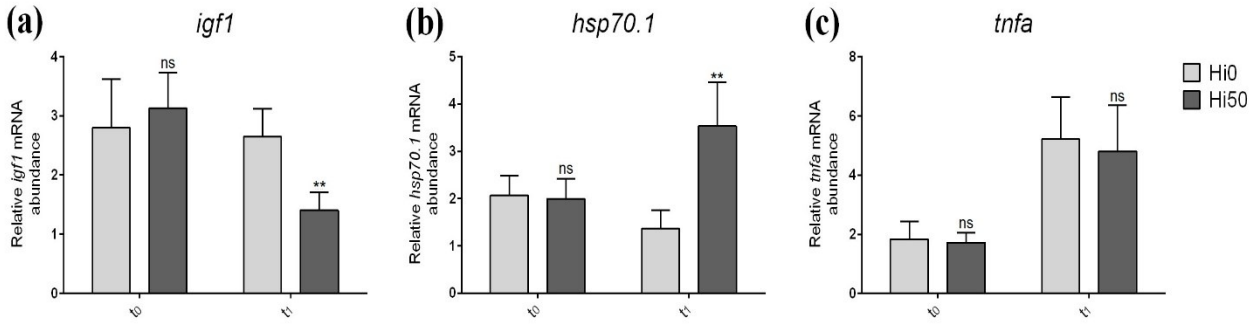


Figure 8. Relative mRNA abundance of genes analysed at t_0 and t_1 in Siberian sturgeon. (**a**) *igf1* and (**b**) *hsp70.1* were analysed in liver samples; (**c**) *tnfa* was analysed in intestine samples. Fish fed diets including 0 and 50 % of BSF prepupae meal (Hi0 and Hi50, respectively) respect to FM. Significant differences between Hi0 and Hi50, compared within the same sampling time, are indicated as follows: *ns* non-significant; * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$ and **** $p < 0.0001$. Values are shown as mean ± SD (n = 5).

Discussion

The inclusion of BSF meal in aquafeed, as well as the physiological responses of fish, have been recently investigated in several important commercial species like Atlantic salmon (*Salmo salar*), rainbow trout (*Oncorhinchus mykiss*) and European seabass (*Dicentrarchus labrax*) [**28**,**30**,**57**]. Information available in this field for the Siberian sturgeon is still fragmentary, and completely lacking if related to aquaponic systems. Previous studies, based on a limited number of laboratory

approaches, highlighted that a defatted BSF prepupae meal dietary inclusion level higher than 25% impaired fish growth and welfare [**31-33**]. Based on these results and previous studies which demonstrated the possibility to use higher full-fat dietary BSF meal inclusion levels [**23**,**38**] after an enrichment procedure of the insect biomass [**39**], the present study aimed to: (i) test, for the first time, a 50% enriched full-fat BSF prepupae meal inclusion level in a practical diet for juvenile sturgeons in an aquaponic system; (ii) apply a set of laboratory techniques to examine comprehensively the physiological responses of the fish. The results showed that the inclusion of a 50% enriched full-fat BSF prepupae meal negatively affects fish growth and survival. This conclusion is supported by the expression of the growth markers analysed and agree with previous studies reporting that inclusion levels of BSF meal higher than 40% often impairs fish growth and welfare [**32**,**36**]. A possible explanation of these differences between the two experimental groups can be related to the fatty acid composition of the diets. Şener et al. [**58**] reported high EPA and DHA levels in Russian sturgeon fed diets rich in linoleic acid. In agreement with this study, our fatty acid analyses showed the ability of sturgeon to convert linoleic acid (18:2n6) and α-linolenic acid (18:3n3) to EPA and DHA by desaturation and elongation enzymatic pathways [**59**,**60**]. Since biochemical conversions require expenditure of energy by the fish, they can explain the observed growth delay in Hi50 compared to Hi0 [**61**].

However, it should be pointed out that a lower diet acceptance was observed in the Hi50 group compared to Hi0 and the laboratory analysis performed suggest that the fish entered a fasting condition. Fasting is normally characterized by a growth reduction mainly due to a decrease in IGFs production by hepatocytes [**62**]. In turn, these changes induce hepatic lipolysis to provide peripheral tissue with free fatty acids as energy source [**62**,**63**]. A similar *scenario* was observed in the present study: Hi50 growth reduction was coupled with a lower *igf1* gene expression and a scarce lipid accumulation in the hepatic parenchyma. These results are also supported by PFF calculation and FTIR analysis which showed an overall decrease in both total lipids (LIP/TBM) and fatty acids (FA/TBM) in the Hi50 liver samples compared to Hi0 ones. Furthermore, a severe reduction of hepatic glycogen, which represents the first energy reserve mobilized to face food restrictions [**64**,**65**], was detected by FTIR in Hi50 compared to Hi0. Furné et al. [**66**] demonstrated that the Adriatic sturgeon (*Acipenser naccarii*) responded to fasting with a precocious mobilization of hepatic glycogen and a high hepatic lipid-degradation capacity. Accordingly, our results represent strong evidence that fish entered a fasting period. In addition, since malnutrition or fasting are nowadays considered stressors [**22**,**67**,**68**], this stressful situation is fully supported by the higher hepatic *hsp70.1* gene expression detected in Hi50 compared to Hi0.

Histological analyses of intestinal tracts are also useful to provide evidence of fasting *status* in fish. Fasting is usually associated to a reduction in mucosal fold number and height, reduction in supra-nuclear lipid droplets and reduction in goblet cells numbers [**69**,**70**]. Accordingly, Hi50 group showed atrophy of mucosal folds and a dramatic decrease of enterocyte vacuolization and goblet cells number compared to Hi0. In a study performed by Caimi et al. [**33**] on Siberian sturgeon over a 118-day period, lower (37.5%) levels of defatted BSF meal dietary inclusion did not show these negative effects on spiral valve and liver histology. Despite the above-mentioned histopathological signs related to fasting, our analyses on gut histology and small intestine *tnfa* gene expression did not reveal any sign of inflammation in either of the experimental groups. Similar results were reported in other studies [**30**,**36**,**71**] that evidenced a positive role of BSF meal dietary inclusion on fish gut welfare. BSF meal contains lauric acid and chitin, that possess anti-inflammatory and immune-boosting properties [**72**]. BSF meal is also known to rise biodiversity in the fish microbiome, necessary to improve fish health, metabolism, nutrition and immunity [**17**,**73**]. Hi50 diet contained higher percentages of lauric acid and chitin and supported a higher number of bacterial groups (at genus or family level) in the gut of fish from Hi50 group compared to those from Hi0, possibly further explaining the absence of intestinal inflammatory events. The dominance of *Mycoplasma,* followed by *Clostridium*, was observed in all samples analysed, regardless of the diet. An increased relative abundance of *Mycoplasma* was detected by Rimoldi et al. [**73**] in the autochthonous gut microbiota of rainbow trout fed with BSF-based diets. This author attributed the beneficial action of this bacterial genus on host health to the production of antibacterial compounds, such as lactic and acetic acids.

In conclusion, the present study demonstrated the feasibility of aquaponic systems for sturgeon culture and for testing new aquafeed ingredients like insect meal. However, the general low feed acceptance showed by the Hi50 group fish compared to Hi0 indicates the need of further studies aimed at improving the palatability of BSF-based diets intended for sturgeon culture.

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4.2

Hermetia illucens and poultry by-product meals as alternatives to plant protein sources in gilthead seabream (*Sparus aurata*) diet: a multidisciplinary study on fish gut status

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Abstract

The attempt to replace marine-derived ingredients for aquafeed formulation with plant-derived ones has met some limitations due to their negative side effects on many fish species. In this context, finding new, sustainable ingredients able to promote fish welfare is currently under exploration. In the present study, poultry by-products and *Hermetia illucens* meals were used to replace the vegetable protein fraction in diets totally deprived of fish meal intended for gilthead seabream (*Sparus aurata*). After a 12-week feeding trial, a multidisciplinary approach including histological, molecular, and spectroscopic techniques was adopted to investigate intestine and liver responses to the different dietary formulations. Regardless of the alternative ingredient used, the reduction in dietary vegetable proteins resulted in a lower incidence of intestine histological alterations and inflammatory responses. In addition, the dietary inclusion of insect meal positively affected the reduction in the molecular inflammatory markers analysed. Spectroscopic analyses showed that poultry by-product meal improved lipid absorption in the intestine, while insect meal induced increased liver lipid deposition in fish. The results obtained demonstrated that both poultry by-products and *Hermetia illucens* meal can successfully be used to replace plant-derived ingredients in diets for gilthead seabream, promoting healthy aquaculture.

Introduction

In the last few decades, the exceptional efforts made in finding novel and sustainable ingredients for aquafeed formulation have caused interest in fish health and physiological responses to the new diets [**1**]. When testing new dietary formulations, an accurate analysis of the organs involved in feed digestion and absorption, immune response, and metabolic processes should be attended. In this regard, the fish gut represents the main target in nutritional challenges, as it plays a primary role in the digestion, absorption, and metabolism of dietary nutrients and local immunity [**2**,**3**]. However, metabolic functions are carried out in synergy with other associated organs. The liver, in particular, plays a primary role in the metabolism of dietary nutrients, and its morphology and tissue composition can be deeply influenced by the diet [**4-7**].

The complex relation between diet, gut integrity, immune response, nutrient assimilation, and liver morphology and composition has been investigated by means of several laboratory techniques [**8**,**9**]. Among these, histology represents the traditional and currently most applied technique used to determine gut responses to dietary challenges based on the analysis of several histo-morphological parameters such as mucosa and sub-mucosa thickness, leucocyte infiltration, and enterocyte lipid vacuolization [**10**,**11**]. In addition, dietary challenges may be able to trigger intestine immune response by the activation of a set of pro- and anti-inflammatory-related genes, which may provide

information on gut health even in the absence of clear histo-pathological evidence [**12**,**13**]. Cytokines such as interleukins and tumor necrosis factor a (tnfa) and inflammation mediators such as nuclear factor kappa-light-chain-enhancer of activated B cells (NF-kB) and myeloid differentiation primary response 88 (MyD88) represent useful markers of inflammation that are able to provide early information when testing new ingredients in aquafeed formulations [**14**,**15**]. To date, besides histological and molecular approaches, target organ responses to dietary challenges were successfully investigated by means of a spectroscopic approach, Fourier Transform Infrared Imaging (FTIRI) spectroscopy, which was shown to be able to provide information on gut intestine absorption and liver biochemical composition in different fish species [**5**,**16**,**17**].

Traditionally, vegetable protein sources have represented a readily available, cost-effective, and advantageous alternative ingredient to fish meal for aquafeed formulation [**18**,**19**]. Among the plant ingredients considered, soybean meal (SBM) represents the most popular vegetable protein source due to its high protein content, optimal amino acid profile, and digestibility [**20**,**21**]. However, SBM is known to exert negative side effects on the gut health and fish welfare of some carnivorous fish species because of its high non-digestible carbohydrate content [**22**,**23**] and the presence of anti-nutritional factors (ANF) [**24-26**]. These negative side effects are particularly known in salmonids [**27**,**28**], while studies on Mediterranean euryhaline cultured fish species such as seabream (*Sparus aurata*) are still scarce and often controversial [**29**]. Besides it has been demonstrated that SBM can be included in seabream diets at relatively high levels [**30**], the activation of non-specific immune response and intestine inflammation occurrence were reported [**31**,**32**]. Similarly, to what has been widely described in salmonids, intestinal morphological changes, including distal intestine leucocyte infiltration and abnormal enterocyte vacuolization, were described in seabream fed a high percentage of SBM [**33**]. Even if in seabream only few studies have analysed the effect of high dietary SBM percentage on immune-related genes in the intestine [**32**,**33**], it is well established that SBM ANF cause gut inflammation by increasing the mRNA levels of pro-inflammatory cytokines in different fish species [**13**]. In addition, the use of high dietary SBM levels has been shown to affect liver morphology and composition in different fish species [**10**,**34**].

For these reasons, finding alternative, sustainable, and cost-effective ingredients that are able to guarantee proper production standards without affecting fish gut health is constantly under exploration [**35**]. Recently, after the authorization by the EC Regulation No. 56/2013 and No. 893/2017, a range of land-produced feedstuff, such as non-ruminant slaughterhouse by-products (named “processed animal proteins”, PAPs) and insects, have received great attention [**36**,**37**]. Among PAP, poultry by-product meal (PBM) represents a highly available, relatively cheap, sustainable, and valued protein source retaining a high nutrient digestibility and a proper essential

amino acids (EAA) profile [**38**,**39**]. However, information about the impact of PBM on fish gut health is still limited [**40-42**]. On the other hand, insect meal has received great attention over the last few years due to its proper amino acidic profile, high protein and mineral content, and low ecological footprint [**43**]. Moreover, the presence of some biologically active compounds, such as chitin, antimicrobial peptides, and medium-chain fatty acids (such as lauric acid), has been shown to improve gut welfare in different fish species [**43-46**].

Recently, PBM [**47-51**] and insect meal [**52**,**53**] were tested as alternative ingredients for seabream diet formulation with promising results in terms of zootechnical parameters, fish health, and fillet quality, while scarce information is so far available about the effects of these ingredients on the fish intestine and liver response. In addition, in the previous experiments, PBM and insect meal were tested as an FM substitute, while the effect of their inclusion on a vegetable-based diet, singly or in combination, is still unknown in gilthead seabream. A proper combination of these different ingredients may represent an interesting and novel approach for the formulation of a new set of sustainable and possibly highly performing fish diets.

On this basis, the aim of the present study was to investigate gilthead seabream (*Sparus aurata*), as one of the most important species in Mediterranean aquaculture, by means of a multidisciplinary approach, assessing its growth performance and gut and liver health in response to diets without fish meal, where graded levels of a blend of vegetable protein-rich ingredients (Vp) were replaced by partially defatted *Hermetia illucens* prepupae meal (HM) or PBM as single ingredients and in combination.

Methods

Ethics

The feeding trial experiment and all the procedures involving animals were carried out in strict accordance with EU legal frameworks relating to the protection of animals used for scientific purposes (Directive 2010/63/EU). It was approved by the Ethics Committee of the University of Udine (Prot. N. 1/2018) and the protocol was authorized by the Italian Ministry of Health (n. 290/2019-PR).

Experimental diets

Seven test diets were formulated to be grossly iso-proteic (45%), iso-lipidic (20%), and isoenergetic (22 MJ/kg). A diet rich in plant-derived ingredients, named CV, was designed to obtain a 90:10 weight ratio between vegetable and marine protein and a 67:33 weight ratio between vegetable and fish lipid, as calculated from the crude protein and lipid contribution to the whole diet of all marine

and plant-based dietary ingredients. Another diet rich in fish meal (CF) was formulated in the opposite way to obtain a 10:90 weight ratio between vegetable and marine protein and a 33:67 weight ratio between vegetable and fish lipid. The remaining diets, named H20, H40, P20, P40, and H10P30, were prepared by replacing graded levels (20 or 40 %) of crude protein from the mixture of vegetable protein sources of the CV diet with crude protein from a commercial partially defatted *Hermetia illucens* prepupae meal (HM) and/or poultry by-product meal (PBM) singly or in combination, while maintaining the same 67:33 vegetable to fish lipid ratio as in the CV diet. The proximate analyses and fatty acid profile of the test ingredients are reported in Table 1.

Table 1. Proximate analysis (%) and fatty acids profile (% fatty acids methyl esters) of commercial *Hermetia illucens* meal (HM) and poultry by-products meal (PBM) used as test ingredients in the present study.

	HM	**PBM**
Proximate composition (%)		
Moisture	4.4	5.8
Crude proteins	53.1	65.6
Ash	6.4	12.4
Chitin	4.69	-
Crude lipids	20.8	14.8
Fatty acids (%)		
SFA	61.08	30.7
12:0	31.8	0.06
14:0	7.1	0.9
18:0	3.3	7.7
MUFA	18.8	48.7
16:1	2.3	5.6
18:1c	13.5	38.8
PUFA	20.2	20.6
PUFA n6	19.1	19.6
18:2n6	18.8	17.3
20:4n6	0.2	1.4
PUFA n3	1.0	1.0
18:3n3	0.9	0.1
20:5n3 (EPA)	-	0.1
22:6n3 (DHA)	0.1	0.1
n6/n3	19.9	19.2

Where necessary, the diets were supplemented with essential amino acids to meet the nutrient requirement of *Sparus aurata* [**54**]. All the diets were manufactured at SPAROS Lda. (Área Empresarial de Marim, Lote C, 8700-221 Olhão, Portugal) by extrusion in two pellet sizes (3 and 5 mm) and stored in a cold room (4 °C) until they were used. The ingredient composition and proximate analysis of the test diets are shown in Table 2. Feed samples were analysed in duplicate for dry matter, DM (AOAC #950.46), crude protein, CP (N × 6.25; AOAC #976.05), and ash (AOAC #920.153) contents according to AOAC International [**55**] and, for the total lipid content, according to Bligh and Dyer, as modified by Burja et al. [**56**].

Table 2. Ingredient (g/100 g) and proximate (% as fed) composition of the test diets.

	CV	H20	H40	P20	P40	H10P30	CF
Ingredient composition							
Fish meals [1]	-	-	-	-	-	-	54.0
Vegetable-protein mix [2]	69.0	52.6	36.6	52.5	35.4	35.4	-
HM [3]	-	16.2	32.4	-	-	8.1	-
PBM [4]	-	-	-	13.8	27.5	20.6	-
Feeding stimulants [5]	5.5	5.5	5.5	5.5	5.5	5.5	5.5
Wheat meal *	0.4	1.6	4.5	3.0	5.6	5.5	3.0
Whole pea *	3.0	5.8	6.0	6.2	9.0	8.8	20.5
Fish oil [6]	6.2	6.2	6.2	6.2	6.2	6.2	8.6
Veg. oil mix [7]	11.4	8.4	5.4	9.8	8.2	7.4	6.5
Vit. & Min. Premix [8]	0.3	0.3	0.3	0.3	0.3	0.3	0.3
Choline HCL	0.1	0.1	0.1	0.1	0.1	0.1	0.1
Sodium phosphate	1.6	1.2	1.0	0.7	0.3	0.2	-
L-Lysine [9]	0.5	0.2	0.2	0.1	0.1	0.1	-
DL-Methionine [10]	0.5	0.4	0.3	0.3	0.3	0.3	-
Celite	1.5	1.5	1.5	1.5	1.5	1.5	1.5
Proximate composition							
Moisture	6.7	6.1	4.7	7.1	7.2	8.7	8.2
Crude protein	45.0	45.2	45.2	45.1	45.1	45.1	45.4
Total lipid	20.4	20.1	20.4	20.4	20.2	20.4	20.3
Ash	5.8	6.6	6.5	7.1	7.9	7.6	12.4
Chitin	0.02	0.76	1.51				

[1] Fish meal mixture (% composition): 26% Pesquera Diamante Peru (66.3% crude ptotein, 11.5% crude fat; 74% Conresa 60, Conserveros Reunidos S.A. Spain (61.2% crude protein, 8.4% crude fat). **[2]** Vegetable-protein source mixture (% composition): dehulled, toasted soybean meal, 39; soy protein concentrate-Soycomil, 20; maize gluten, 18; wheat gluten, 15; rapeseed meal, 8. **[3]** ProteinX™, Protix, Dongen, The Netherlands (55.4% crude protein; 20.8% crude fat). **[4]** Poultry by-product meal from Azienda Agricola Tre Valli; Verona, Italy (65.6% crude protein; 14.8% crude fat). **[5]** Feeding stimulants g/100 diet fish protein concentrate: CPSP90- Sopropeche, France (82.6% crude protein), 3.5; Squid meal (80.3% crude protein), 2.0. **[6]** Fish oil: Sopropêche, France. **[7]** Vegetable oil mixture (% composition): rapeseed oil, 56; linseed oil, 26; palm oil, 18. **[8]** Vitamin and mineral supplement (per kg of premix): Vit. A, 2,000,000 IU; Vit D3, 200,000 IU; Vit. E 30,000 mg; Vit. K3, 2500 mg; Vit.B1, 3000 mg; Vit. B2, 3000 mg: Vit B3, 20,000 mg; Vit. B5, 10,000 mg; Vit B6, 2000 mg, Vit. B9, 1500 mg; Vit. B12, 10 mg, Biotin, 300 mg; Stay C®, 90,000 mg; Inositol, 200,000 mg; Cu, 900 mg; Fe, 6000 mg; I, 400 mg; Se, 40 mg; Zn, 7500 mg. **[9]** L-lysine, 99%; Ajinomoto EUROLYSINE S.A.S; France. **[10]** DL-Methionine: 99%; EVONIK Nutrition & Care GmbH; Germany. * Wherever not specified, the ingredients composing the diets were obtained from local providers by Sparos Lda. # Estimated based on the chitin content of the ingredients used (squid meal, 0.9%, and *Hermetia illucens* meal, 4.69%).

Fish rearing conditions, calculation and sampling

Three-hundred seventy-eight gilthead seabream juveniles (initial mean body weight 48.8 ± 8.8 g) were selected to be uniform in size from a resident stock of 1200 specimens. Fish were divided among 21 cylindrical fiberglass tanks with a capacity of 300 L (18 individuals in each tank) fitted with a device to recover unfed pellets. Tanks were connected to a marine recirculating aquaculture system supplied with sand mechanical and biological filters, a protein skimmer, an ozonator, and an UV lamp (Scubla srl, Remanzacco, Udine, Italia), which ensured the optimal water quality (water temperature, 23.4 ± 0.75 °C; salinity, 31 ± 0.7 g/L; dissolved oxygen, 5.9 ± 0.22 mg/L; pH, 8.0 ± 0.1; TAN < 0.02 mg/L; NO_2^- < 1.0 mg/L). During the feeding trial, fish were kept under a constant day length and

intensity (12 h per day at 400 lux) provided by fluorescent light tubes. Fish groups were left to adapt to the culture conditions over two weeks before being randomly assigned in triplicate to the seven dietary treatments. Fish were fed by belt feeders (Scubla srl), six days a week in two daily meals (8:00 am and 4:00 pm) slightly in excess to satiety over twelve weeks. To this end, each tank was also fitted with an apparatus for recovering uneaten feed pellets shortly after being released by the feeder. Excess satiety was attained by distributing a daily feed amount adjusted to exceed the intake of the previous day to obtain feed residues after each meal. The feed amounts distributed were recorded daily and uneaten feed was recovered, dried, and weighed to estimate the actual feed intake.

At the end of the feeding trial, after a 24 h fasting period (adopted in order to avoid excess of feed in gut, which could interfere with laboratory analysis), all the fish were subjected to stage 3 anaesthesia with 80 ppm of MS-222 (PHARMAQ Ltd., Fordingbridge, Hampshire, UK) and individual biometry measurements (total length and body weight) were recorded. Subsequently, three fish per tank (9 fish per dietary treatment) were sacrificed with an overdose (300 ppm) of the same anaesthetic and liver and medium and distal intestine were sampled and properly stored for histological, molecular, and spectroscopic (FTIR) analyses, as described in the following sections.

At the end of feeding trial for each tank-specific growth rate (SGR), the relative feed intake (RFI) and feed conversion ratio (FCR) were calculated as follows: SGR = 100 × [(ln final body weight - ln initial body weight) / days]; RFI (g/kg/ABW/d) = feed intake per tank / [(initial biomass + final biomass) / 2) / days] were ABW means average body weight; FCR = feed intake per tank / weight gain per tank.

Histological analyses

Samples were prepared according to Randazzo et al. [**16**]. Briefly, samples (n = 9 for each dietary group) from liver and medium and distal intestine were fixed in Bouin's solution and stored at 4 °C for 24 h. Samples were then washed and preserved in 70% ethanol solution. After dehydration by graded ethanol solutions, samples were washed with xylene (Bio-Optica, Milan, Italy) and embedded in solid paraffin (Bio-Optica). Paraffin blocks were cut with a microtome (Leica RM2125RTS, GmbH, Wetzlar, Germany) and 5 µm sections were stained with Mayer haematoxylin and eosin Y (Merck KGaA, Darmstadt, Germany). Stained sections were examined under a Zeiss Axio Imager.A2 (Zeiss, Oberkochen, Germany) microscope and the images were acquired by means of a combined colour digital camera Axiocam 503 (Zeiss). A semi-quantitative evaluation of the distal intestine morphology, based on mucosal fold height, mucosal fold fusion, enterocyte supranuclear vacuoles, and sub-mucosa width was performed, as previously described by Urán et al. [**57**] and Zarantoniello et al. [**17**] (chapter 4.1). Specifically, for the intestinal fold morphometric evaluation, ten transversal

sections of the medium and distal intestine at 200 µm intervals for each sample were analysed. All the undamaged and non-oblique folds (at least 150 measurements per fish) were measured using the ZEN 2.3 software (Zeiss), and results were reported as the means of the observations. On the same sections, a semi-quantitative analysis of the histopathological indexes was performed. For the histopathological index scores, an arbitrary unit was assigned for each parameter, as described in Panettieri et al. [**58**]. The sections were analysed by experienced personnel in two independent blinded evaluations and the score assignment criteria are described in Table 3.

Table 3. Semi-quantitative scoring system for the different parameters used as histopathological indexes of enteritis in gilthead seabream intestine.

	Score	Description
Mucosal fold fusion (MF f)	+	0-5 observations per section
	++	5-15 observations per section
	+++	> 15 observations per section
Supranuclear vacuoles (SnV)	+	Scarce
	++	Diffused in the enterocytes
	+++	Abundantly filling enterocytes
Sub-mucosa width (SM w)	+	10-15 µm
	++	15-30 µm
	+++	> 30 µm

In order to evaluate the percentage of fat fraction (PFF) in the liver, three sections for fish (n = 9) for each experimental group at 100 µm intervals were acquired and analysed by means of the ImageJ software, setting a homogeneous threshold value according to Zarantoniello et al. [**44**] (chapter 3.1). Areas that were not evaluable on the sections, such as blood vessels and bile ducts, were not considered. The results were reported as the percentage of the area occupied by fat on the total hepatic parenchyma analysed on the section.

Fourier Transform Infrared Imaging (FTIRI) spectroscopy measurements and data analysis

Nine samples of liver and medium and distal intestine were collected for each dietary group and immediately stored at -80 °C. To perform Fourier Transform Infrared Imaging (FTIRI) measurements, for each sample three sections (10 µm thick) were cut using a cryotome 200 µm apart from each other. Sections were deposited without any fixation process onto CaF_2 optical windows (1 mm thick, 13 mm diameter) and then left to air-dry for 30 min [**59-61**]. FTIRI measurements were carried out by means of a Bruker Invenio interferometer coupled with a Hyperion 3000 Vis-IR microscope and equipped with a Focal Plane Array (FPA) detector operating at liquid nitrogen temperature (Bruker Optics, Ettlingen, Germany). On each section, by means of a 15× condenser/objective, specific areas were detected on which the IR maps were acquired in transmission mode in the Mid-InfraRed (MIR) range (4000-800 cm^{-1}; spectral resolution 4 cm^{-1}; 128 scans). Before

each acquisition, the background spectrum was acquired on a clean portion of the CaF_2 optical window. All the raw IR maps were pre-processed using the Atmospheric Compensation (to correct for the atmospheric contributions of carbon dioxide and water vapor) and Vector Normalization (applied on the full frequency range to avoid thickness variations) routines (OPUS 7.5 software package, Bruker Optics).

Distal and medium intestine. The FTIRI analysis was performed on the intestinal folds; each IR map was 164 × 328 μm in size and was composed of 8192 pixel/spectra with 2.56 × 2.56 μm as the spatial resolution. False colour images representing the topographical distribution of lipids, fatty acids, proteins, and glycosylated compounds were created by integrating pre-processed IR maps under the following spectral regions: 3000-2800 cm^{-1} (representative of total lipids, LIP maps), 1700-1480 cm^{-1} (representative of proteins, PRT maps), and 1190-1120 cm^{-1} (representative of glycosylated compounds, COH maps). From each IR map, 200 IR spectra were extracted in the outermost layer of intestinal folds to evaluate the biochemical composition of the absorbent mucosa of this compartment. On these IR spectra, the following band area ratios were calculated in relation to the total biological mass (TBM) and statistically analysed: LIP/TBM (ratio between the integrated area of the 3000-2800 cm^{-1} region, representative of total lipids, and TBM, calculated as the sum of the 3000-2800 cm^{-1} and 1780-900 cm^{-1} integrated areas), PRT/TBM (ratio between the integrated area of the 1700-1480 cm^{-1} region, representative of total proteins, and TBM, calculated as described above), and COH/TBM (ratio between the integrated area of the 1190-1120 cm^{-1}, representative of glycosylated compounds, and TBM, calculated as described above) (Integration routine, Mode B, OPUS 7.5 software package).

Liver. The analysis was performed on liver sections of all experimental groups, without choosing any region, due to the homogeneity of this tissue. IR maps were 164 × 164 μm in size and were composed of 4096 pixel/spectra with a spatial resolution of 2.56 × 2.56 μm. False colour images representing the topographical distribution of total lipids, unsaturated lipids, fatty acids, proteins, and glycogen were created by integrating pre-processed IR maps under the following spectral regions: 3000-2800 cm^{-1} (representative of total lipids, LIP), 1700-1480 cm^{-1} (representative of proteins, PRT), and 1080-1000 cm^{-1} (representative of glycogen, GLY). From each IR map, the following band area ratios were calculated and statistically analysed: LIP/TBM (ratio between the area of the 3000-2800 cm^{-1} region, representative of total lipids, and TBM, calculated as described above), PRT/TBM (ratio between the area of the 1700-1480 cm^{-1} region, representative of total proteins, and TBM, calculated as described above), and GLY/TBM (ratio between the area of the 1080-1000 cm^{-1} region, representative of glycogen, and TBM, calculated as described above).

RNA extraction and cDNA synthesis

Samples were prepared according to Olivotto et al. [**62**,**63**]. Briefly, the total RNA was extracted from the medium and distal intestine samples (n = 9 for each experimental group, approximately 90 mg per sample) using the RNAzol RT reagent (Merck KGaA) and following the manufacturer's instructions. The RNA concentration and integrity were analysed using NanoPhotometer P-Class (Implen, Munich, Germany) and Gel Red™ (Merck KGaA) staining of 28S and 18S ribosomal RNA bands on 1% agarose gel, respectively. After extraction, complementary DNA (cDNA) was synthesized from 1 µg of total RNA with the LunaScript RT SuperMix Kit (New England Biolabs, Ipswich, MA, USA), following the manufacturer's instructions, diluted 1:10 in RNase-DNase-free water, and stored at -20 °C until use. An aliquot of cDNA was used to check the primer pair specificity.

Real-time PCR

The mRNA levels of the selected genes involved in immune response (intestine) - namely, interleukin-1β (*il1b*), interleukin-10 (*il10*), tumor necrosis factor alpha (*tnfa*), nuclear factor kappa-light-chain-enhancer of activated B cells (*nfkb*), myeloid differentiation primary response 88 (*myd88*), and toll-like receptor-1 (*tlr1*) - were assessed. The primers sequences were retrieved from NCBI (http://www.ncbi.nlm.nih.gov/ accessed on 26 January 2021) and are summarized in Table 4. Amplification products were sequenced, and the homology was verified. Negative controls revealed no amplification product, and no primer-dimer formation was found in the control templates. PCRs were performed according to Piccinetti et al. [**64**] and Vargas et al. [**5**] in an iQ5 iCycler thermal cycler (Bio-Rad, Hercules, California, USA), and each sample was analysed via RT-qPCR in triplicate. Reactions were set on a 96-well plate by mixing, for each sample, 1 µL of cDNA diluted 1:20, 5 µL of 2× concentrated iQ™ Sybr Green (Bio-Rad) as the fluorescent intercalating agent, 0.3 µM of forward primer, and 0.3 µM of reverse primer. The thermal profile for all reactions was 3 min at 95 °C, followed by 45 cycles of 20 s at 95 °C, 20 s at specific annealing temperature (for details, please see Table 4), and 20 s at 72 °C. Fluorescent signal were detected at the end of each cycle and melting curve analysis was performed to confirm that only one PCR product was present in these reactions. For the gene expression relative quantification, beta-actin (*b-actin*) and ribosomal protein S18 (*rps18*) RNA were used as housekeeping genes to standardize the results. Data were analysed using the iQ5 optical system software version 2.0, including Genex Macro iQ5 Conversion and Genex Macro iQ5 files (all from Bio-Rad).

Table 4. Sequences, annealing temperature (A.T.) and Gene Bank ID of the primers used for the present study.

Gene	Forward Primer (5'-3')	Reverse Primer (5'-3')	A. T. (°C)	Gene Bank ID
il1b	ATCCAGCTGTCTTTCCCTCA	TTGCATGTCATCTCGGATTC	59	XM_030435228.1
il10	AACATCCTGGGCTTCTATCTG	TGTCCTCCGTCTCATCTG	60	JX976621.1
tnfa	CTGTGGAGGGAAGAATCGAG	CTTTCTGGTCCACCTCACCT	60	XM_030412624.1
nfkb	GTTTGTCGTGTCGTTGGGAG	CGAGTGGACAAGTGAGTGGA	58	XM_030403588.1
myd88	CCGTCGTCTGTGGCTAACAT	GTCCCACGCCTTTTTCAACC	56	XM_030399037.1
tlr1	CTTGTGCCCAGCAGTGTTTC	CGGTTTGTAGCACGGTCTTC	60	XM_030396315.1
β-actin	TCCTGCGGAATCCATGAGA	GACGTCGCACTTCATGATGCT	57	X89920.1
rps18	AGGGTGTTGGCAGACGTTAC	CTTCTGCCTGTTGAGGAACC	57	AM490061.1

Statistical analysis

Growth performance data are expressed as means ± standard deviation (SD). Data were checked for normal distribution and homogeneity of variance before analysis, and growth parameters were subjected to a one-way analysis of variance (ANOVA). When significant differences were detected, the Tukey's multiple-comparison test was used to assess the differences among groups. Differences were considered significant when $p < 0.05$. Analyses were carried out using the SPSS-PC release 17.0 (SPSS Inc., Chicago, IL, USA). For the histological and gene expression statistical data analyses, the Graph software package Prism5 (Graph Pad Software, La Jolla, CA, USA) was used. Histological measurements, IR, and gene expression results were reported as mean ± SD and were analysed through a one-way ANOVA with a Tukey's multiple-comparison test for the comparison of the means; the level of significance was set at $p < 0.05$.

Results

Growth performances

Fish promptly accepted all the test diets and no mortality occurred throughout the trial. The growth performance of seabream after the 12-week trial is shown in Table 5. The fish fed P20, P40, H40, and H10P30 diets resulted in a similarly higher growth performance than those of fish fed CV and CF diets, while fish fed H20 diet showed an intermediate value. Fish fed CF diet resulted in a higher feed consumption when compared to the other dietary treatments, which did not significantly differ from each other. As a consequence of the reduced growth and increased feed intake, the fish fed CF diet exhibited the worst FCR relative to all the other dietary groups, but this was similar to that observed for the fish fed CV diet.

Table 5. Growth performance, specific growth rate (SGR), relative feed intake (RFI), and feed conversion ratio (FCR) of gilthead seabream kept at 23.4 ± 0.75 °C and fed the test diets over 12 weeks.

Dietary treatments	Final weight g/fish	SGR	RFI g/kg ABW/d	FCR
CV	177.7 ± 2.10 [a]	1.54 ± 0.01 [a]	15.9 ± 0.21 [a]	1.18 ± 0.05 [b]
H20	187.5 ± 3.60 [b]	1.59 ± 0.02 [b]	15.8 ± 0.16 [a]	1.13 ± 0.04 [a]
H40	192.2 ± 0.80 [c]	1.64 ± 0.02 [c]	15.6 ± 0.07 [a]	1.11 ± 0.04 [a]
P20	191.6 ± 1.47 [c]	1.63 ± 0.01 [c]	15.9 ± 0.33 [a]	1.11 ± 0.03 [a]
P40	192.3 ± 0.36 [c]	1.63 ± 0.01 [c]	15.8 ± 0.38 [a]	1.10 ± 0.02 [a]
H10P30	190.7 ± 1.32 [bc]	1.62 ± 0.01 [c]	15.7 ± 0.08 [a]	1.11 ± 0.02 [a]
CF	180.1 ± 1.41 [a]	1.55 ± 0.02 [a]	16.4 ± 0.08 [b]	1.21 ± 0.02 [b]

Intestine and liver histology

Intestine histology showed a variable degree of morphological alteration in both intestine tracts analysed in all the experimental groups, except for CF (Figures 1a and 2a). A remarkable intensification of the main alterations observed was detected in both the medium and distal intestine from CV group. Particularly, the histological alterations included oedema (Figures 1b and 2b), enterocyte hyper-vacuolization (Figure 1c), submucosa thickening (Figure 1d), mucosal fold fusion (Figure 2c), and basal inflammatory influx (Figure 2d).

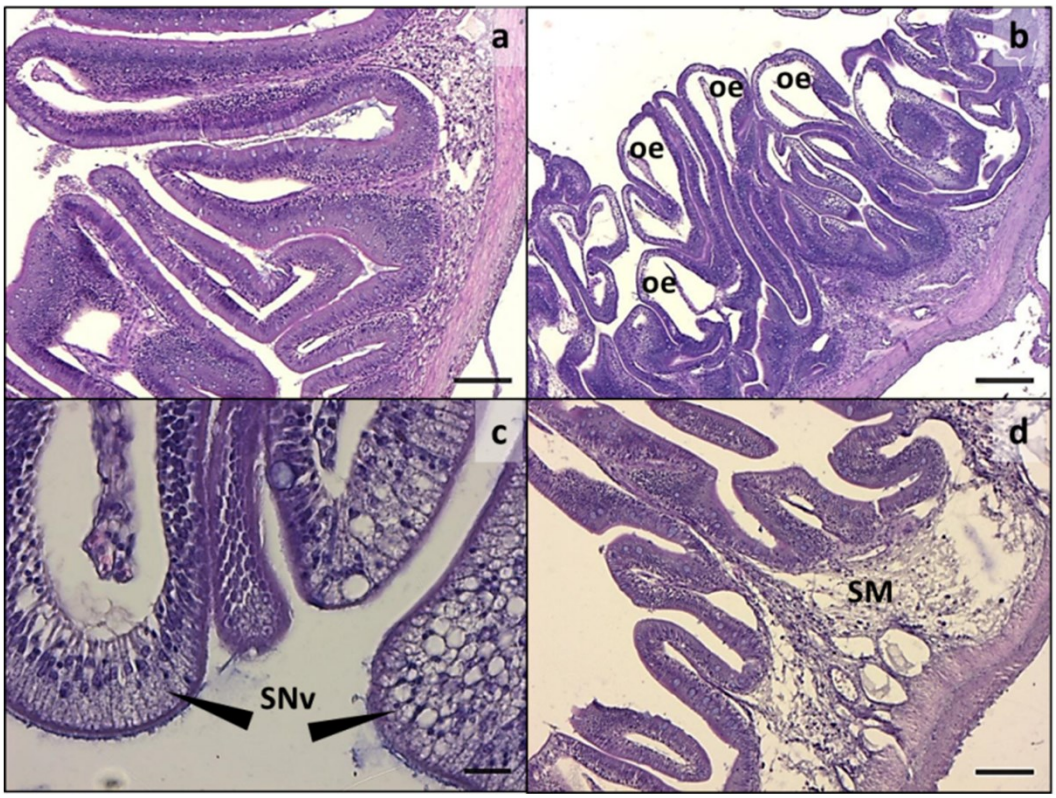

Figure 1. Medium intestine. (**a**) Normal histology from CF group; (**b**) histological architecture alteration, including oedema and severe folds fusion degree in CV group; (**c**) high magnification showing enterocyte hyper-vacuolization in CV group; (**d**) highly infiltrated and vacuolated submucosa in CV group. Letters: oe = oedema; SNv = supranuclear vacuoles; SM = submucosa. Scale bars: (**a**,**d**) 100 µm; (**b**) 200 µm; (**c**) 20 µm.

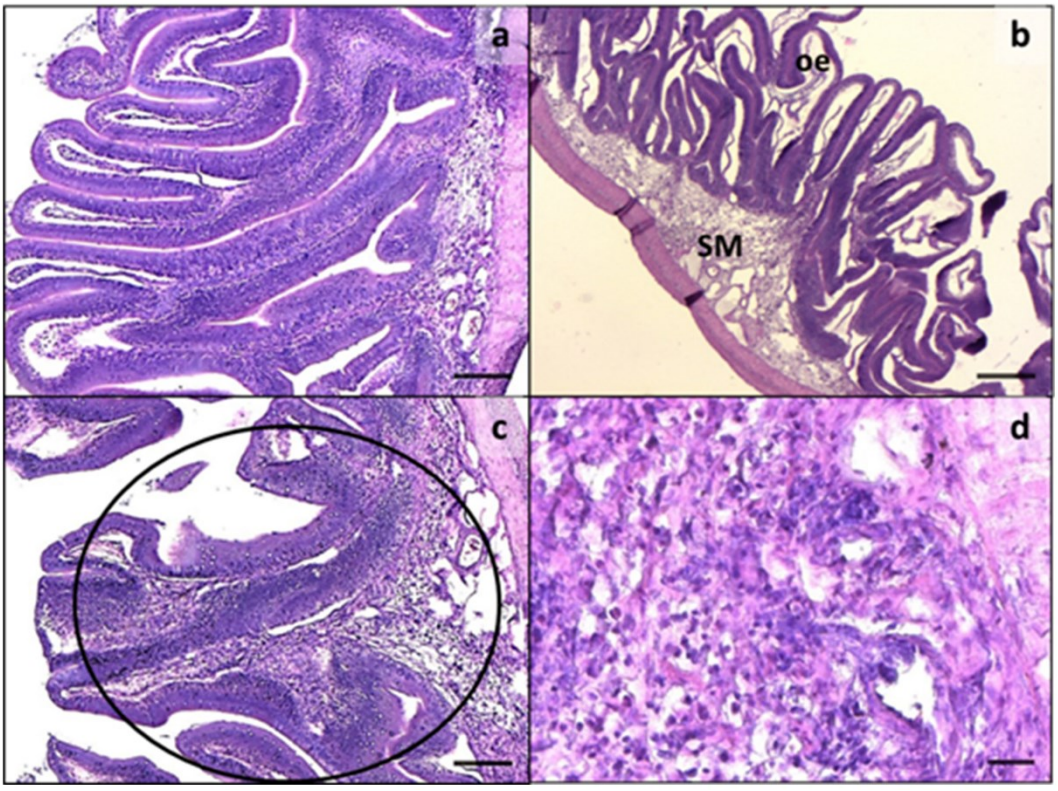

Figure 2. Distal intestine. (**a**) Normal histology from CF group; (**b**) histological architecture alterations, including diffuse oedema (oe) and submucosa thickening in CV group; (**c**) detail of fold fusion (circle) in CV group; (**d**) high magnification showing lymphocyte infiltration in CV submucosa. Letters: SM = submucosa. Scale bars: (**a**,**c**) 100 µm; (**b**) 200 µm; (**d**) 20 µm.

Table 6. Mucosal fold morphometric evaluation and histological index scores in the medium and distal intestine from fish fed experimental diets.

	Medium intestine				Distal intestine			
	MF (µm)	**MF f**	**SN v**	**SM w**	**MF (µm)**	**MF f**	**SN v**	**SM w**
CV	853 ± 12 ^{a}	+++	+	++	631 ± 79 ^{a}	+++	+++	+++
H20	1003 ± 88 ^{b}	+	+	+	672 ± 97 ^{a}	+	++	+
H40	1092 ± 88 ^{b}	+	++	+	813 ± 15 ^{b}	+	+	+
P20	1010 ± 12 ^{b}	+	++	+	956 ± 94 ^{b}	+	+	+
P40	1023 ± 11 ^{b}	+	++	+	846 ± 59 ^{b}	+	+	+
H10P30	1073 ± 23 ^{b}	+	+++	+	948 ± 95 ^{b}	+	+	+
CF	1100 ± 13 ^{b}	+	+++	+	821 ± 80 ^{b}	+	+	+

Letters: MF = mucosal fold height; MF f = mucosal fold fusion; SN v = supranuclear vacuoles; SM w = submucosa width. Mucosal fold height is expressed by the means of the measurements performed ± SD. Different superscript letters indicate significant differences among the experimental groups ($p < 0.05$).

The mucosal fold morphometric evaluations and histological index scores are reported in Table 6. Comparing all groups, a significant reduction in fold height was observed in the medium intestine of fish fed CV diet and in the distal intestine of those given CV and H20 diets with respect to the other experimental groups. A significantly higher incidence of fold fusion was also observed in the medium and distal intestine of fish fed CV diet compared to the other groups. Enterocyte supranuclear

vacuolization was significantly increased in the medium intestine of fish given H40, P20, P40, H10P30, and CF diets compared to those fed CV and H20 ones. On the contrary, a higher incidence of supranuclear vacuolization was observed in fish fed CV diet compared to all the other dietary groups. Finally, only CV diet resulted in an inflammatory influx, causing a significant submucosa thickening in the medium and distal intestine (Figures 1d and 2b-d).

Representative images of the liver histological sections from all the experimental groups are shown in Figure 3. All the analysed samples presented a compact parenchyma with abundant lipid deposition in all the experimental groups. No differences in the percentage of lipid deposition were found by PFF analysis (Table 7)

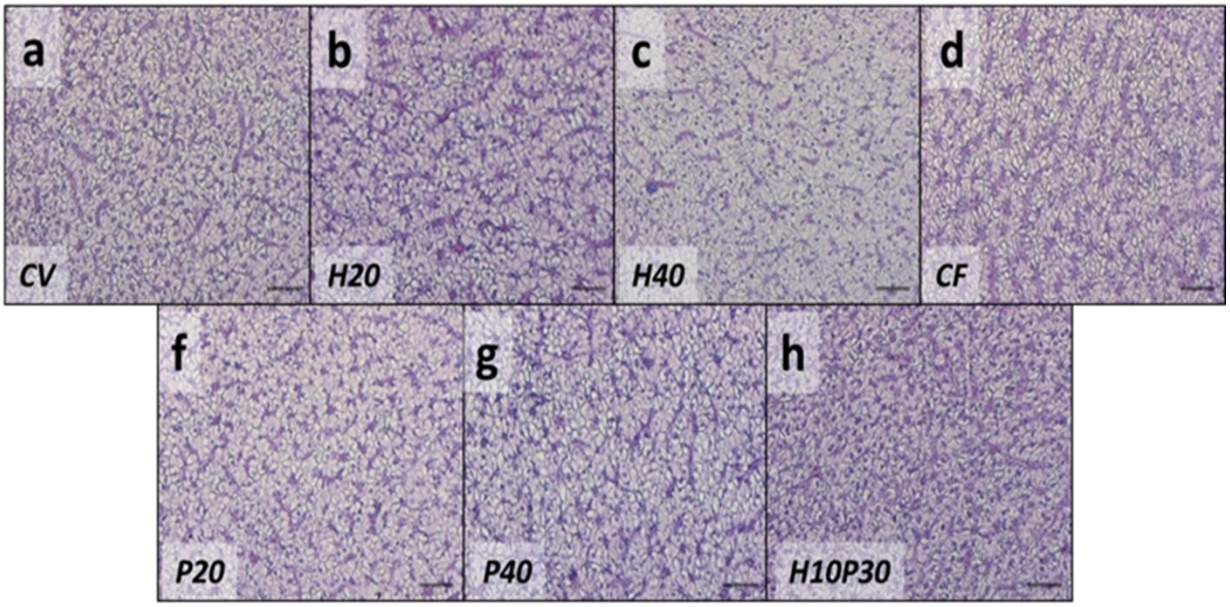

Figure 3. Representative histological images of liver parenchyma from fish fed the experimental diets: (**a**) CV; (**b**) H20; (**c**) H40; (**d**) CF; (**f**) P20; (**g**) P40; (**h**) H10P30. Scale bars = 100 µm.

Table 7. Histological evaluation of the percentage of fat fraction (PFF) in liver histological sections.

	PFF
CV	58.8 ± 1.8
H20	63.5 ± 2.2
H40	65.6 ± 2.2
P20	61.7 ± 1.3
P40	63.1 ± 2.3
H10P30	62.9 ± 2.3[c]
CF	62.4 ± 1.4

Data are reported as mean ± SD. No significant differences were detected among groups.

Gene expression

The gene expression analysis performed on medium and distal intestine tissue samples showed a diet-related modulatory effect on inflammatory and immune molecular markers. In the medium intestine (Figure 4a), a significant overexpression of *il1b* was observed in the fish fed CV diet, while a significant downregulation was detected in those given H40, P40, and H10P30 diets compared to the other groups. A similar trend according to the different diets was also observed for the gene expression of *il10*, but it was not significant. In the same intestinal tract, a significant downregulation of *nfkb* was observed in fish fed H20 and H40 diets, while no significant differences were observed among the other groups. On the contrary, the gene expression of *tnfa*, *myd88*, and *tlr1* was unaffected by dietary treatments.

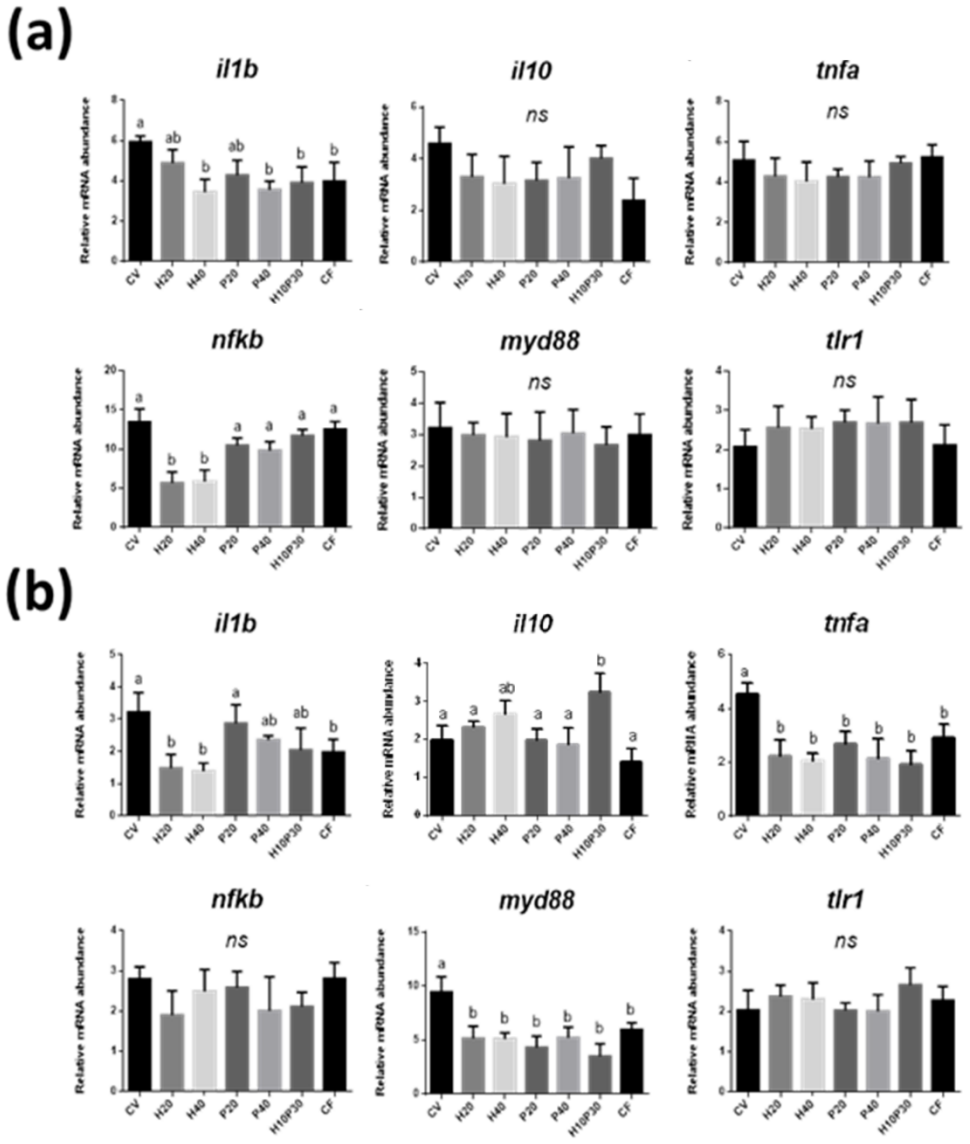

Figure 4. Relative mRNA abundance of genes involved in inflammation and immune response in the (**a**) medium and (**b**) distal intestine. Values are presented as mean ± SD. Different letters indicate significant differences among the experimental groups ($p < 0.05$); *ns* = not significant differences.

In the distal intestine (Figure 4b), the magnitude of differences in gene expression due to dietary treatments was more marked. As in the medium tract, *il1b* was overexpressed in fish fed CV diet compared to those given CF one, with this latter not significantly differing from the gene expression observed in fish given H20 and H40 diets. Fish fed P20 diet did not differ from those fed CV one, while all the diets including PBM showed intermediate *il1b* gene expression between those of fish fed the control diets. In fish fed H10P30 diet, the *il10* gene expression was higher compared to all the other dietary groups, except for H40 which resulted in intermediate values. The gene expression of *tnfa* and *myd88* was similarly downregulated in fish fed CF diet or all ones including test ingredients (H20, H40, P20, P40, H10P30) when compared to fish fed CV diet. No differences among dietary groups were observed in the *nfkb* and *tlr1* gene expression, similarly to what was observed in the medium intestine.

FTIRI analysis

Fourier Transform Infrared Imaging (FTIRI) spectroscopy was used to characterize the macromolecular composition of the medium and distal intestinal mucosa and liver parenchyma. Thanks to false colour images, the topographical distribution of the macromolecules in the intestine and liver samples level was analysed.

Distal and medium intestine. The hyperspectral imaging analyses of representative sections of CF medium and distal intestine samples are shown in Figure 5a and b, respectively. False colour images showed the topographical distribution of total lipids (LIP maps), proteins (PRT maps), and glycosylated compounds (COH maps). In both intestinal tracts, the main macromolecules considered were predominantly detected at the level of the outermost layer of intestinal folds, which represents the intestine absorbent portion. To evaluate changes in the biochemical composition of the absorbent portion of intestinal mucosa in relation to the different diets, the following band area ratios were analysed: LIP/TBM (relative amount of total lipids), PRT/TBM (relative amount of proteins), and COH/TBM (relative amount of glycosylated compounds). In the medium intestine (Figure 6a): (i) H20 and H40 samples showed lipid values similar to CV (LIP/TBM), while significantly higher values in P20, P40, H10P30, and CF samples were detected; (ii) no statistically significant differences were found among all groups with regard to proteins (PRT/TBM); (iii) CV, P20, P40, H10P30, and CF exhibited the same amount of glycosylated compounds (COH/TBM), while significantly higher values were observed in H20 and H40. In the distal intestine (Figure 6b), significantly higher lipid values were found in CV and H20 compared to the other groups. No significant differences were found among all groups with regard to proteins (PRT/TBM) as well as glycosylated compounds (COH/TBM).

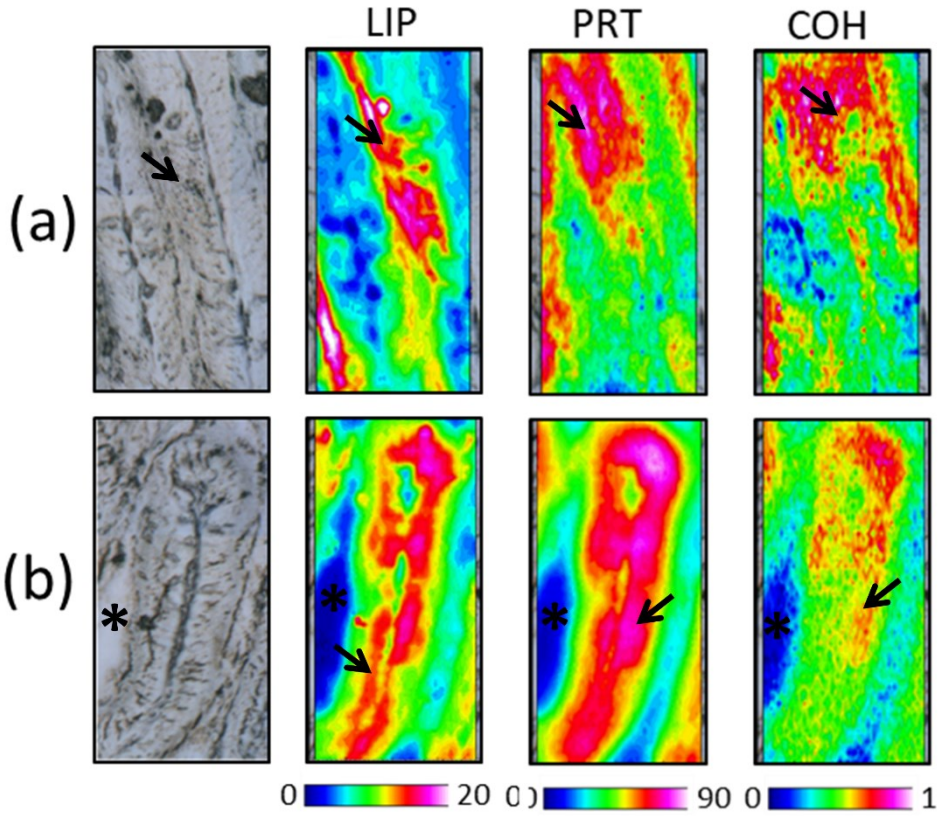

Figure 5. Hyperspectral imaging analysis of representative sections of (**a**) medium and (**b**) distal intestine of CF dietary group. False colour images (164 × 328 µm) showing the topographical distribution of: lipids (LIP, 0-20 colour scale), proteins (PRT, 0-90 colour scale), and glycosylated compounds (COH, 0-1 colour scale). Black/dark blue colours represent the lowest absorbance values of the infrared radiation, while white/light pink represent the highest ones. Arrows indicate the mucosa epithelial layer; asterisks indicate the intestinal lumen.

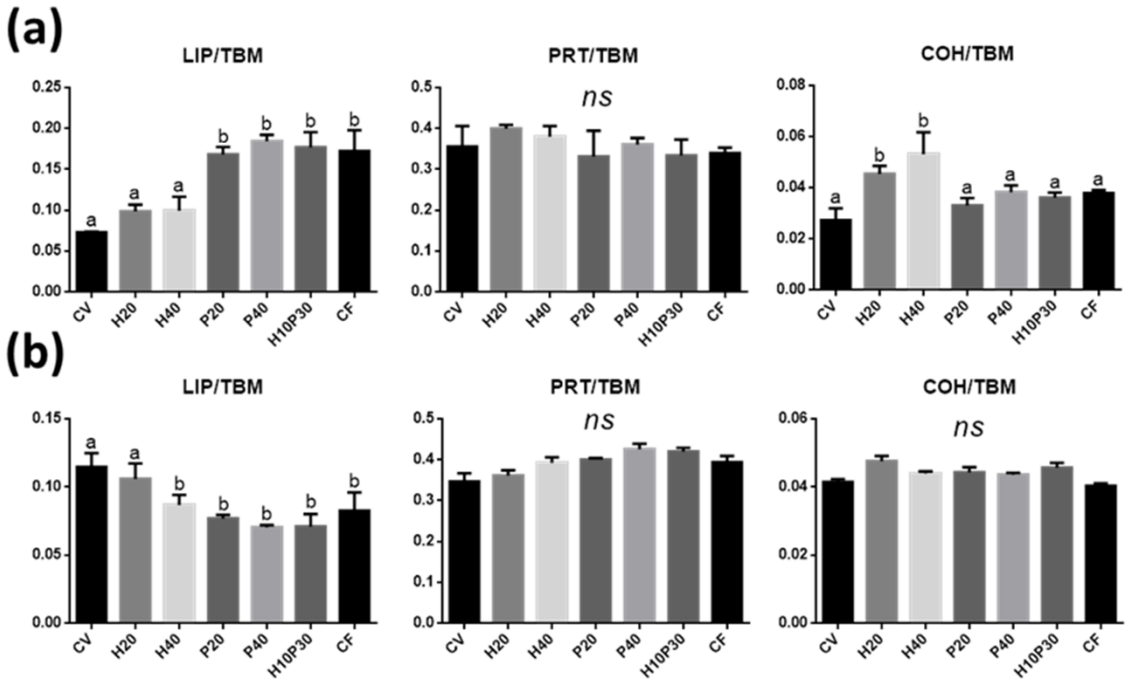

Figure 6. Biochemical composition of (**a**) medium and (**b**) distal intestine mucosa. Statistical analysis of the following band area ratios: LIP/TBM (relative amount of total lipids), PRT/TBM (relative amount of total proteins), and COH/TBM (relative amount of glycosylated compounds).

Liver. The hyperspectral imaging analysis of a representative liver section from CF group is shown in Figure 7. The false colour images showed the topographical distribution of total lipids (LIP map), proteins (PRT map), and glycogen (GLY map). As expected, based on the structural features of the liver, all the analysed macromolecules appeared to be homogeneously distributed within the mapped areas. Nevertheless, an almost impossible distribution was observed for the total lipids and glycogen, which was similar to that displayed by proteins.

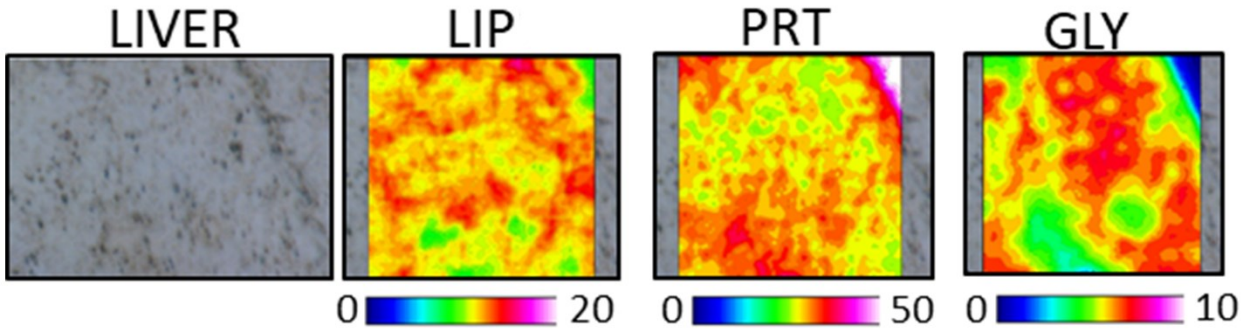

Figure 7. Hyperspectral imaging analysis of a representative liver section from CF group. False colour images (164 × 164 µm) representing the topographical distribution of total lipids (LIP, 0-20 colour scale), proteins (PRT, 0-50 colour scale), and glycogen (GLY, 0-10 colour scale). Black/dark blue colour represents the lowest absorbance values of infrared radiation, while white/light pink represent the highest ones.

To evaluate changes in the biochemical composition of liver parenchyma in relation to the different diets, the following band area ratios were analysed (Figure 8): LIP/TBM (relative amount of total lipids), PRT/TBM (relative amount of proteins), and GLY/TBM (relative amount of glycogen). With regard to lipids (LIP/TBM), similar contents were observed in all groups, except for H40 which showed significantly higher values compared to the other groups. No statistically significant differences were found among all groups with regard to proteins (PRT/TBM) and glycogen (GLY/TBM).

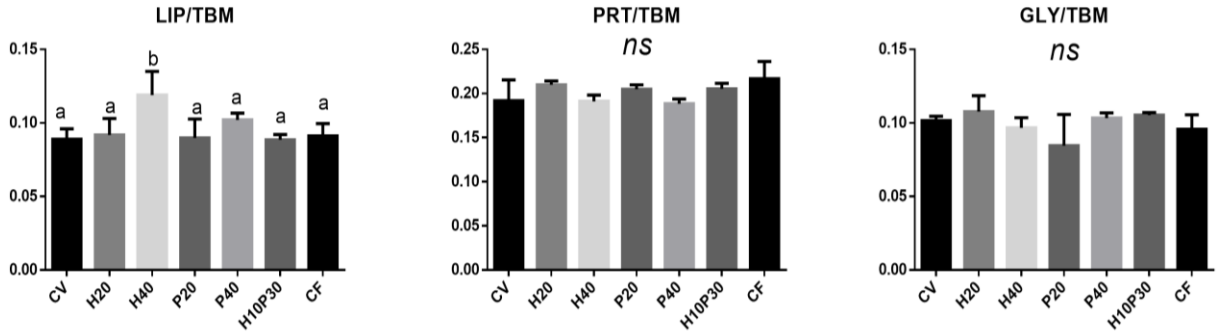

Figure 8. Biochemical composition of liver. Statistical analysis of the following band area ratios: LIP/TBM (relative amount of total lipids), PRT/TBM (relative amount of total proteins), and GLY/TBM (relative amount of glycogen).

Discussion

Previous studies demonstrated that gilthead seabream is able to tolerate high levels of dietary FM substitution, either by vegetable or animal protein sources [**10**,**65**,**66**]. However, even if with less extent compared to other carnivorous fish species, moderate adverse side effects on the digestive system of this species were observed when substantial levels of plant-derived ingredients were used in diets [**10**,**31**,**33**,**67-69**]. Accordingly, in the present study, the onset of moderate intestine histopathological changes in fish fed a diet in which vegetable protein-rich ingredients (Vp) represented the major protein source (CV) enforces the evidence that plant-derived ingredients represent only a partial alternative to replace FM in diets for gilthead seabream. On the contrary, reducing dietary Vp resulted in improved histological gut conditions, including a reduction in the main intestine morphological changes observed in the CV group.

To date, besides protein-rich plant derivatives, other protein sources such as PAPs and, recently, insect meals have been tested in diets for gilthead seabream [**70-72**]. Among PAPs, poultry by-product meal has been studied in previous experiments on juvenile sea bream, where it was found not to be detrimental in terms of growth performance when included up to 36% in diets largely based on FM [**51**]. Additionally, HM has been studied as a novel protein source in diets for gilthead seabream. In juveniles, growth, feed efficiency, and nutrient retention were not reduced when HM was included to replace up to 30% of dietary FM protein [**73**].

In the present study, PBM and HM inclusion resulted in excellent zootechnical performances, in line with the outcomes of earlier studies, even if the results obtained are not readily comparable mainly because in previous investigations PBM and HM were tested in diets where FM represented the main protein source. Differently, in the present study, their inclusion occurred in spite of a Vp in diets nearly deprived of FM. Irrespective of the inclusion level in the diet, both test ingredients resulted in improved growth and feed conversion ratios compared to both control diets. A better outcome with diets including PAPs relative to the CV diet could depend on a better overall amino acid balance and reduced levels of antinutritional factors due to reduced levels of Vp as PBM and/or HM, singly or in association, were included in the diet. On the other hand, improved growth performance and efficiency with diets including alternative protein sources compared to the FM-based control diet has already been described in gilthead sea bream [**67**]. Moreover, in the present study, this could be also partially ascribed to a reduced nutrient and energy digestibility of CF diet compared to the test ones due to its high proportion of fair (low)-quality FM [**74**].

To the best of our knowledge, little is known about the effects of PBM and HM on gilthead seabream gut physiology. In the present study, replacing Vp with PBM or HM (singly or in combination) resulted in no appreciable negative histopathological change in the liver and digestive tract compared

to a FM-based diet (CF), demonstrating the tolerance of gilthead seabream at the levels of dietary inclusion investigated. While histological inspection is widely used to provide a reliable picture of intestine condition, the analysis of a set of molecular markers of inflammation is commonly used to obtain deeper information on gut response to dietary challenges. In particular, cytokines are immune-modulating agents acting as pro-inflammatory mediators (*il1b* and *tnfa*) or playing an important role in the adaptive immunity response, as in the case of *il10* [**75**]. The upregulation of cytokines and other molecular mediators has been observed as a common response to low FM-based diets in European seabass [**76**]. In this regard, Estruch et al. [**68**] demonstrated that a long-term feeding period (22 weeks) with a Vp-based diet led to changes in the inflammatory and immune-related gene expression at the intestinal level in gilthead seabream. The results of the present trial showed that the activation of inflammatory cascade due to high levels of Vp (SBM in particular) composing the CV diet can be observed in a shorter period (12 weeks), leading to the overexpression of some molecular markers such as *il1b*, *nfkb*, *tnfa*, and *myd88*, particularly in the distal intestine. On the contrary, a general downregulation of inflammatory markers was observed when the Vp dietary content was reduced. Considering that improved gut conditions in the present trial were observed regardless of the alternative ingredient used (replacing Vp), especially in the distal intestine, the results can easily be related to a reduction in dietary ANF characterizing SBM [**77**], which represented the major protein source in the Vp blend. Nevertheless, the molecular results showed a more significant inflammatory marker downregulation in fish fed diets including HM, as highlighted by the *il1b* and *il10* gene expression in the distal intestine of fish fed H20 and H40 diets. Recent studies have demonstrated that an inclusion of HM up to 21% in low FM diets was able to downregulate inflammatory response in rainbow trout [**6**]. Furthermore, HM has been reported to have beneficial effects on gut physiology in different cultured fish species [**78**], mainly attributed to the presence of certain bio-active compounds, such as chitin and medium-chain fatty acids, exhibiting immuno-stimulating, antimicrobial, and/or anti-inflammatory properties [**43-46**]. Fish tolerance to chitin (and its derivatives) varies among fish species [**79**], and no studies are available on the in vivo effects of dietary HM inclusion on gilthead seabream gut health. However, in vitro experiments suggest that chitin can enhance cellular immune activity in leucocytes from fish species, even if the mechanisms involved in immune system modulation remain unclear [**80**]. Furthermore, medium-chain fatty acids, such as lauric acid (highly represented in HM), have been associated with anti-inflammatory and immune-boosting properties [**81**-**82**], providing a further explanation for the inflammation processes mitigation observed in the intestine of fish fed H20 and H40 diets. However, other possible roles of the fatty acids composition of the diets besides lauric acid cannot be ruled out. In fact, replacing vegetable proteins with HM and PBM possibly resulted in changes in the composition of dietary

lipids which went beyond the different proportions between fish and vegetable oils. This could have played a role in the physiological response observed. With all test diets being iso-lipidic, such putative effects might be related to a different dietary fatty acid composition/ profile due to a different lipid contribution of the test ingredients to the overall dietary fatty acid profile. This could have resulted in changes in the n6/n3 PUFA ratio in diets including different levels of PBM compared to those including HM with potential effects on the balance between pro- and anti-inflammatory eicosanoids. Besides analysing the effects of PBM and HM on gut health, the aim of the present study was to investigate the intestine nutrient absorption and the liver biochemical composition in response to the test diets. In previous studies, FTIR analysis was successfully applied in fish intestine, providing a reliable picture of the nutrient absorption in rainbow trout [**83**]. On this basis, in the present study, the hyperspectral results obtained by FTIR analysis detected an improved lipid absorption in the medium intestine in response to a dietary replacement of Vp with HM and PBM. Plant-derived ingredients, particularly SBM, are known to lower lipid absorption and retention in fish tissues [**84-86**], thus providing a possible explanation for the improved lipid absorption observed in the medium intestine when dietary Vp were reduced. However, the modulation of lipid absorption here observed in the medium intestine suggested a different role of HM and PBM, with the latter resulting in increased lipid absorption, as observed in groups fed P20, P40, and H10P30 diets compared to H20 and H40 ones. To some extent, this could depend on the different unsaturation degree of the lipid fraction of the two test ingredients. It is well known that lipid absorption and digestibility appear negatively correlated to the dietary inclusion level of saturated fatty acids [**87**], which are notably higher in the lipid fraction of HM compared to PBM. A different scenario was observed in the distal intestine. Indeed, the higher values of total lipids detected in this tract in fish fed CV diet should not be interpreted as improved lipid absorption, since lipids are mainly absorbed in the proximal intestine [**88**,**89**]. Lipid abundance shown by FTIR analysis in this tract of the intestinal mucosa could result from the abnormal enterocyte hyper-vacuolization observed by histological analysis. Lipid enterocytes vacuolization represents a physiological condition in the fish proximal and medium intestine, while it has been considered as a pathological sign in the distal intestine of different fish species, including gilthead seabream [**90**,**91**]. It should be noted that this condition was markedly reduced in fish fed CF diet as well as diets including intermediate or high levels of the test ingredients. However, further investigation are needed in this regard.

Additionally, the FTIR analysis of the medium intestine showed a dose-dependent increase in glycosylated compounds (representative of carbohydrates) in fish fed graded dietary levels of HM compared to all the other dietary treatments. This could be a result of chitin degradation and absorption, suggesting the ability of gilthead seabream to digest this polysaccharide [**92**].

In the present study, liver histological analysis did not show appreciable differences in lipid accumulation, while FTIR revealed a higher lipid deposition in fish fed H40 diet. Considering that no differences regarding protein and glycogen content were highlighted in the livers from fish fed the different experimental diets, the higher liver lipid content in fish from the H40 group can be related to the different HM fatty acids profile, known to be rich in saturated and monounsaturated fatty acids rather than polyunsaturated ones [**93**].

Overall, the results obtained in the present study demonstrated that HM and PBM can be successfully used to replace Vp in a vegetable-based diet for gilthead seabream without negatively affect fish growth performances and welfare. In addition, HM showed interesting effects ameliorating the fish intestine condition, while PBM exerted beneficial effects on intestine nutrient absorption. These alternative ingredients can be used to counteract the negative side effects due to the high amount of plant ingredients in diets for gilthead seabream.

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Chapter 5

Overview of the available laboratory approaches to study the physiological responses of fish to BSF-based diets

5.1

Application of laboratory methods for understanding fish responses to Black Soldier Fly (*Hermetia illucens*) based diets

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Abstract

A major challenge for development of sustainable aquafeeds is its dependence on fish meal and fish oil. Replacement with more sustainable, nutritious and safe ingredients is now a priority. Over the last years, among several alternatives proposed, insects have received great attention as possible candidates. In particular, the black soldier fly (*Hermetia illucens*; BSF) represents a concrete example of how the circular economy concept can be applied to fish culture, providing a valuable biomass rich in fat and protein valorising organic by-products. In the last decade, several studies have been published about the use of different BSF dietary inclusion levels for various fish species including experimental models. Varying and encouraging results have been obtained in this research field using a plethora of laboratory methodological approaches that can be applied and coupled to obtain a comprehensive view of the BSF-based diets effects on fish physiology, health, and quality. The present review aims to explore some of the most promising laboratory approaches like histology, infrared spectroscopy, gut microbiome sequencing, molecular biology, fish fillets' physic, chemical and sensory properties, essential for a better understanding of fish welfare and fillet quality, when BSF is used as aquafeed ingredient. In particular, great importance has been given to European finfish species and experimental models.

Introduction

Due to the global capture fisheries stagnation, aquaculture is presently the fastest growing food production sector worldwide [**1**]. Over the last years, there has been a significant increase in worldwide consumption of aquatic products [**2**] which is expected to further expand over the next thirty years [**3**]. In this context, the aquaculture industry will face an important challenge: providing a world population estimated to reach 9.7 billion by 2050 with a proper amount of nutritious and safe aquatic food [**2**,**4**]. In order to meet this challenge, aquaculture production has to grow further [**1**]. Although there is a high market demand for fish, a further development of aquaculture poses serious environmental challenges. As a result of ecological impacts of aquaculture inputs and resources, including water, land and feed, the sustainability in aquaculture is often restricted by a wide range of environmental concerns [**5**,**6**]. Today, sustainable aquaculture technology and know-how have significantly advanced, and they can play a pivotal role in achieving environmental sustainability goals. Long-term growth of the aquaculture industry must be based on the concept of environmentally friendly practices and sustainable resource management. Presently, after the launch of Horizon 2020 by the European Community, aquaculture priorities can be summarised in three keywords: safe, sustainable, responsible. These words represent the basis for better animal's welfare, less

environmental impact, and the development of new approaches able to provide larger volumes of healthy and safe food.

Aquaculture feed accounts for 50-70% of production costs [**7**] and represent an equal share of the CO_2 footprint [**8**]. Because of the over-exploitation of pelagic fisheries, strategies to replace fish meal (FM) and fish oil (FO) in aquafeeds became both a private and public priority [**9**]. The study for low cost and sustainable feeding alternatives to replace totally or partially FM and FO have gained relevance [**10**,**11**] and to help the aquaculture industry to expand and remain competitive. Alternative ingredients are, for example vegetable proteins, including oilseeds (especially soybeans), and meat by-products (such as blood meal and bone meal). Among others, plant meals (PM) are the most widely used alternatives to FM since 2006 [**12-14**]. However, PM have unbalanced essential amino acids profile (due to the deficiency in methionine and lysine), low protein content, anti-nutritional factors and a significant amount of non-digestible carbohydrates, which often limit their use for carnivorous fish [**15**,**16**]. In particular, non-digestible carbohydrates, including non-starch polysaccharides, may bind to bile acids or obstruct the action of digestive enzymes as well as feed transit in the intestine reducing both nutrients digestibility and absorption [**17**]. In addition, PM can cause inflammation of the digestive tract of fish [**10**,**18**] but also show low palatability [**10**,**19**]. Over the years, several techniques have been developed to partially overcome these issues including the use of enzymes, heat processes and the inclusion of bioactive compounds in the formulated diets [**12**,**20**]. In recent years, PM registered a significant rise in prices because of its increased use in human nutrition, causing a competition between the animal feed and human food sectors [**21**]. As a consequence, PM industry has limited potential to expand its production without putting additional pressure on arable land use and water consumption and shifting resource demand from oceans to land [**22**].

Another alternative ingredient to FM in aquaculture is animal by-product meal (ABP). These meals contain a good balance of essential amino acids, show high protein content and good digestibility with features similar to those of FM [**23-25**]. Although the ABP seems to be a good and viable alternative, at technical and economic level, the consumer acceptance, a strict regulation and many restrictions for their use due to a lack of knowledge on the risk for developing human diseases are its main limitation [**26**]. In the European Union, the use of ABP was prohibited from 1990 to 2000 due to the arising of the bovine spongiform encephalopathy in ruminants and then, in 2013, was allowed only for ABP derived from non-ruminant animals (Category 3) [**27**]. European legislation states that Category 3 ABP can be processed for feeding aquatic animals to contribute responsibly to both environment and public health [**28**].

Recently, the circular economy concept has gained great attention within the European Community and the EC Directive No. 2008/98 [**29**], which establishes the order of importance in the choice of

by-products treatment (the first being their recycle and the last being their landfill disposal) is playing a key role.

In order to meet the circular economy concept, aquafeed production can take advantage of the great amount of organic by-products which can be converted in a valuable biomass rich in proteins and lipids, by using bio-converting organisms [**30-33**]. Specifically, bioconversion through insects grown on organic by-products as feed source might represent a valuable solution [**10**,**34**,**35**]. It also represents a valid example of sustainable animal production in terms of land use, water consumption and CO_2 production, because of the low energy requirements during rearing procedures [**36**,**37**]. The nutritional values of the insects' biomass are generally characterised by high fat and protein content [**34**,**38**]; however, the insects' biomass nutritional composition depends on the quality and quantity of feed offered for their growth [**32**,**33**,**39**,**40**], with the fat content and quality varying the most (from 7 to 39 % dry matter) [**32**,**41**].

Among the species of insects used for aquafeed production, *Hermetia illucens* (L.) (Diptera, Stratiomydae) (black soldier fly; BSF) is one of the most promising species: it shows high efficiency as bio-converter of organic by-product during the larval development [**42**], a proper protein content (up to 60% dry weight) and an essential amino acid pattern similar to that of FM [**43**,**44**]. Furthermore, BSF larvae represent a good source of lipids usually dominated by saturated fatty acids (SFA) (especially rich in lauric acid, 12:0), a medium-chain fatty acid which has anti-inflammatory and immune-boosting properties [**45**,**46**]. Their high content in SFA rather than in polyunsaturated fatty acid (PUFA) still represent a key-issue to be solved since this unbalanced fatty acids profile could impair fish growth, welfare and quality [**47**].

Recently, methods to modulate the final biomass' nutritional profile by enriching the growing substrate have been developed [**48**,**49**]. In particular, Truzzi et al. [**32**] demonstrated that growing BSF prepupae on a rearing substrate enriched with 10% *Schizochytrium* sp. can improve insect's final biomass, especially in terms of some fatty acids like PUFA.

The inclusion of new ingredients in aquafeeds must be approached cautiously, since it is well-known that modulatory effects of different feed ingredients on fish physiological responses, quality and safety exist [**50**,**51**]. The scientific community should take into consideration productivity, fish welfare and end-product's quality in order to guarantee both animals and humans wellbeing.

While productivity is not affected by proper insect meal inclusion levels in aquafeeds [**52**], the main problem found in the use of insect meal in fish production is changes in the fatty acids profile of the fish product [**53**]. When fed on insect meal based diets, fish fillets generally showed a reduced n3/n6 ratio, as well as low PUFA content, reducing the nutritional quality of fish for human consumption [**54**,**55**]. In addition, since a large variety of organic by-products can be used as feed for insects,

potentially toxic elements and pathogen microorganisms can enter the food production chain and be potentially harmful to animals and humans [**56**,**57**]. Therefore, to meet a safe production of insects, a strict chemical and microbiological monitoring is necessary.

During the last decade, the issue of farmed fish welfare has raised increasing public and scientific concern [**58**]. Animal welfare has been defined as 'the aptitude of an animal to familiarise to its environment and maintain good health, while living a natural life and show its natural behaviour' [**59**,**60**]. Much research on the welfare of farmed fish is thus required to provide recommendations for best practices and future legislation [**59**,**61**]. Farmed fish are usually exposed to a variety of stressors including handling, stocking density and nutrition which long-term exposure could have negative effects on health and growth performances [**62-65**]. When it comes to new feed ingredients the gastrointestinal tract plays a key role. It constitutes an important barrier to the external environment [**66**], providing defence against pathogens and tolerance to dietary antigens [**67**] while playing, at the same time, a fundamental role in the absorption of nutrients [**68**] and on the innate and adaptive immunity of fish [**69**,**70**]. Therefore, intestine integrity is considered to be essential to sustain a proper fish growth and welfare.

Presently, the fast development of science and the development of several new laboratory techniques allow scientist to have a deep and comprehensive approach when studying the effects of new practical diets in aquaculture. Numerous laboratory techniques like histology, infrared spectroscopy, gut microbiome sequencing, molecular biology as well as fish fillets' physic-chemical and sensory properties are now available for a better understanding of the above-mentioned aspects related to fish farming, representing an up-to date approach for a better understanding of the effects of insect-based diets on fish welfare and production.

The present review aims to provide an overview on the main analytical methods presently available for understanding welfare and quality of fish when fed on diets including BSF meal. Emphasis is given to a number of fish species of interest for the European aquaculture as well as to experimental models.

Fish gut and liver

Histology

Novel feed formulations for fish need to evaluate not only economical cost/benefit but also the effect on fish welfare. The gastrointestinal system is the primary target of dietary changes and challenges [**71**].

Histological analysis of the digestive apparatus is considered one the main approaches to evaluate fish welfare and nutritional status [**72**]. In particular, intestine and liver are the most important organs

involved in digestive and immune functions and, consequently, are of particular interest when new ingredients are applied to aquafeed formulation [**73-75**]. Intestinal morphology is considered one of the main indicators of fish health since its morphological structure rapidly and often reversibly changes in response to dietary inputs. Alteration of gut integrity may modify nutrient absorption and thus fish welfare and growth, possibly affecting productivity [**11**,**76-80**]. Morphometric assessment of intestine architecture is widely used for studying intestine response to dietary challenges and involves the measurement of a number of histopathological parameters, in fish species [**81-85**]. Histological analysis relies on the use of tissue staining techniques to visualise intestinal morphology and specific cell markers. Among the most widely used stainings, haematoxylin and eosin staining (H&E) is commonly applied to provide general morphological information and to detect and discriminate inflammatory cells (lymphocytes, granulocytes, melanomacrophages, etc.) [**86**] based on their acidophilic (eosin) or basophilic (haematoxylin) features.

Other stainings allow to obtain more accurate information about the chemical composition of some cell categories, by virtue of specific chemical reactions [**87**]. As for example, periodic-acid Schiff (PAS) staining and Alcian blue pH2 (AB) staining are elective for neutral and acid (PAS and AB, respectively) mucins in mucous cells [**88**,**89**]. The analysis of mucous cells in the intestinal epithelial layer is also of particular interest [**90**,**91**]. These cells are able to produce and release defensive substances including mucins, lectins, toxins, immunoglobulins and antimicrobial peptides in response to specific dietary stimuli or mechanical injury [**92-94**].

Aside providing information on the cell type and tissue morphology, histological analysis can be useful to obtain a number of histopathological parameters able to provide (semi-) quantitative information on possible intestinal alterations through a multi-grade scoring system [**95**].

The most traditional histopathological indexes include mucosal folds morphology (length, width, fusion), enterocytes supranuclear vacuolisation, *lamina propria* and submucosa width, leucocyte infiltration (also named ‘cellularity’), and mucous cells abundance [**68**,**78**,**81**,**96-98**]. Since insect meal is known to include different molecules like chitin and medium-chain fatty acids, which may have an important role in gut welfare regulation [**45**,**51**,**99**], the analysis of all these parameters has recently been applied to several studies in order to provide information on possible inflammation and/or alterations in the nutrient transport in fish [**100**].

Most of the histological studies have been performed on Atlantic salmon (*Salmo salar*) [**50**,**101**], rainbow trout (*Oncorhynchus mykiss*) [**90**,**102**] and zebrafish (*Danio rerio*) [**33**,**103**], while a few studies are available on Siberian sturgeon (*Acipenser baerii*) [**91**,**104-106**] and Japanese sea bass (*Labrax japonicas*) [**107**].

As regards Atlantic salmon, studies which adopted histological analyses in support to other laboratory techniques, showed that a partial (40%) up to a total substitution of FM with full-fat [**101**] or partially defatted [**100**] BSF prepupae meal did not cause negative effects on post-smolt Atlantic salmon intestine morphology, indicating a high tolerance of salmonids to high dietary BSF meal inclusion levels.

The inclusion of a full-fat BSF prepupae meal in pre-smolt Atlantic salmon diet showed beneficial effects on fish intestine by reducing the enterocyte hyper-vacuolisation which usually characterises intestine of fish fed diets including high levels of soybean meal [**50**,**108**] and decreased steatosis in proximal intestine [**100**]. Moreover, studies performed on rainbow trout showed that up to a 50% dietary substitution of FM with partially defatted BSF prepupae meal did not affect anterior or distal intestinal tracts morphology [**102**,**109**]. However, Józefiak et al. [**110**] showed a slight decrease of villi height in trout proximal intestine fed a 50% dietary full-fat BSF inclusion level with respect to FM. In another recent study performed on rainbow trout fed diets containing 25 or 50 % full-fat BSF meal with respect to FM, no intestine severe inflammatory events were highlighted but a significant mucosal fold height reduction was observed in both groups, respected to control [**90**]. In addition, Randazzo et al. [**111**,**112**] showed a dose dependent increase of distal intestine mucous cells in rainbow trout fed diets in which 30 or 60 % of vegetable proteins were replaced with defatted BSF prepupae meal. This result suggests a possible involvement of undigested chitin in inducing a higher lubrication of the intestine final tract. Similarly, it was demonstrated that dietary administration of full-fat BSF prepupae meal (25 or 50 % with respect of FM) stimulated a higher secretion of neutral mucins rather than the acidic ones, along the entire digestive tract of rainbow trout [**90**]. These results suggest a possible involvement of undigested chitin in inducing a higher lubrication of the intestine final tract. Differently, Elia et al. [**113**] did not evidence significant differences in mucous cells (both rich in neutral and acidic mucins) in rainbow trout, independently of partially defatted BSF larvae meal dietary inclusion (25 or 50 %). However, the role of chitin in inducing mucous cells proliferation has still not been directly demonstrated and further studies are necessary.

To date, no studies are available on the effects of BSF meal dietary inclusion on European sea bass (*Dicentrarchus labrax*) intestinal histology, while one has recently been published on gilthead seabream (*Sparus aurata*) by Randazzo et al. [**114**]. In addition, a recent study on Japanese sea bass showed that a replacement up to 64% of FM by defatted BSF meal did not affect intestine integrity [**107**]. Only recently, a few studies were focused on the effects of BSF prepupae meal dietary inclusion on Siberian sturgeon gut morphology: Józefiak et al. [**106**] showed that a diet in which 15% of FM was replaced by full-fat BSF meal caused a reduction in intestinal muscular and mucosal thickness but did not affect mucosal folds length, while Caimi et al. [**104**] did not report histological alterations

of the distal intestine in fish fed highly defatted BSF meal (25 or 50 % replacement with respect to FM). Conversely, Zarantoniello et al. [**91**], in juvenile sturgeons, demonstrated that a 50% dietary inclusion level of full-fat BSF meal with respect to FM induced mucosal folds atrophy and a dramatic decrease of enterocyte vacuolisation.

Recently, the effects of the administration of BSF-based diets in the experimental model zebrafish were deeply investigated. Particularly, a general increase in mucous cells number was observed in zebrafish larvae fed exclusively on full-fat BSF prepupae meal [**115**]. On the contrary studies in which zebrafish were fed on diets including increasing inclusion levels of full-fat BSF prepupae meal (with respect to FM) did not show intestine morphology changes [**33**,**103**,**116**].

In addition to intestine, liver is often considered a second target organ when testing new aquafeed ingredients. This is particularly important when testing insect meal since its fatty acids composition, rich in SFA, may alter the lipid accumulation in this organ [**117**]. Liver welfare is a key aspect for fish production since this organ plays a central role in many of the fish metabolic pathways and its morphological structure and macromolecular composition are deeply influenced by the diet [**90**,**117-119**].

Several histological analyses, using the above-mentioned histological stainings, may be applied to the liver, providing information about lipid accumulation, inflammation, necrosis, and glycogen deposition in this organ. McFadzen et al. [**120**] proposed a criteria scale for the analysis of fish liver in order to determine the nutritional conditions and this approach has been successfully applied over the years for studying the effects of dietary challenges on farmed fish species [**121**]. Liver histological parameters are responsive to food quality and availability [**122**] and are particularly addressed to provide information on hepatocyte morphology, with emphasis on the relative amount of intracellular vacuolisation associated with lipid and glycogen storage.

The fatty acids profile of insects does not always match the nutritional requirements of fish since insects are rich in medium-chain SFA and MUFA rather than in long-chain PUFA. Liver lipid and glycogen deposition can provide important information on the effects and suitability of BSF-based diets [**90**,**118**,**123**]. While traditional staining (H&E) allows to detect hepatocytes lipid storage, histochemical PAS staining helps in detecting liver glycogen accumulation [**124**,**125**].

In post-smolt Atlantic salmon fed full-fat BSF prepupae meal diets (from 33 up to 100 % with respect to FM), a dose-dependent increase in liver lipid accumulation was observed [**35**], while only minor effects on liver lipid deposition were observed in Japanese sea bass fed diets including defatted BSF prepupae meal [**107**]. Similarly, an increase in liver lipid deposition was evidenced in rainbow trout fed diets containing 25 or 50 % full-fat BSF meal with respect to FM [**90**,**118**].

Studies performed on zebrafish suggested that the n6/n3 ratio is a key factor in determining hepatic lipid accumulation. The higher the ratio value, the higher was the hepatic lipid accumulation [**33**,**103**,**115**,**126**]. Finally, Li et al. [**127**] evidenced a reduction in liver lipid deposition in the herbivorous species Jian carp fed on defatted BSF-based diets.

With respect to glycogen deposition, to date, only a few studies applying a histological approach are available. PAS was used to discriminate the contribution of glycogen in hepatocytes composition of juvenile clownfish (*Amphiprion ocellaris*) fed partially defatted BSF larvae meal (25, 50 or 75 % replacement with respect to FM) and rainbow trout fed full-fat BSF prepupae meal (25 or 50 % with respect of FM). However, no significant differences among the experimental groups were detected in terms of glycogen accumulation [**90**,**117**].

From this overview, it is clear that fish responses are not only species-specific but also dependent on fish life stage and on the characteristics of BSF meal (full-fat or defatted). A traditional and well-established method like histology plays a pivotal role in the analysis of fish gut and is still used as gold standard serving scientific research because of its validity and accuracy. These types of analysis could provide the community with essential insights on the effects of BSF meal on fish.

Fourier transform infrared spectroscopy: hyperspectral imaging analysis and biomolecular composition

Fourier Transform Infrared (FTIR) spectroscopy is a well assessed analytical tool for the analysis of biological samples, such as tissues, cells and biological fluids [**128-130**]. The interaction between the electromagnetic radiation in the mid-infrared spectral range (4,000-800 cm^{-1}) and the samples causes vibrational transitions at the level of chemical bonds, allowing to identify the presence of specific chemical groups [**131**]. This potentiality coupled with the optical microscopy allows to perform the hyperspectral imaging analysis of selected areas inside the analysed samples. In addition, this tool lets combine the topographical distribution of the most relevant biomolecules (in terms of lipids, proteins, carbohydrates and nucleic acids) with meaningful information on the biochemical composition and the occurrence of specific biological mechanisms and pathways [**71**,**116**,**132**].

FTIR spectroscopy has recently been applied to evaluate the physiological responses of fish species to BSF-based diets, including rainbow trout, Siberian sturgeon, and zebrafish. Most of the studies applying this specific technique have been performed on zebrafish, where administration of diets with increasing full-fat BSF meal levels (0, 25, 50, 75 or 100 % with respect to FM; Hi0, Hi25, Hi50, Hi75 and Hi100 fish groups, respectively) was tested during the larval, juvenile and adult phases. As regards the zebrafish larval phase, due to the small sample size, FTIR analysis was performed on lyophilised samples. Statistically significant higher amounts of overall lipids and SFA were found in

Hi50, Hi75 and Hi100 groups with respect to control (Hi0) and Hi25, together with a decrease of the unsaturated fatty acids. Conversely, no significant changes were detected in terms of protein composition [**33**]. In juvenile and adult zebrafish, cryosections (10 μm thick) of specific target organs (such as liver and ovaries) were analysed. In liver, the hyperspectral imaging analysis provided details on the biochemical composition of this organ which were subsequently coupled to the histological data to provide a more comprehensive overview [**91**,**116**]. As an example, liver samples of juvenile zebrafish fed diets including increasing BSF meal levels (0, 25, 50, 75 or 100 % with respect to FM; Hi0, Hi25, Hi50, Hi75 and Hi100 groups, respectively) showed an increment of total fatty acids together with a decrease of both carbohydrates and phosphates. Specifically, in Hi75 and Hi100 liver samples, higher amounts of total lipids and lower ones of proteins were detected, while the lowest glycogen levels were found in Hi50 and Hi75 groups [**116**].

Recently FTIR was used to characterise the macromolecular composition of class IV oocytes of zebrafish adult females fed over a 12-month period on BSF-based diets [**132**]. Specifically, while similar amounts of SFA, MUFA and PUFA were found in control and Hi25 groups, Hi50 showed higher SFA and lower PUFA contents. This study suggested that the substitution of FM with BSF meal up to 25% in zebrafish female diet did not affect reproductive performance, while a higher inclusion (50% with respect to FM) resulted in reproductive impairments, specifically in terms of number of spawned eggs.

Since high dietary SFA and n6 PUFA intake have often been related to behavioural and cognitive impairments in humans and rodents, FTIR was also applied to better understand the fatty acids composition of zebrafish brain in response to dietary BSF meal inclusion. Although specific behavioural tests such as open-field and photic entrainment tests did not evidence differences among the experimental groups, the FTIR analysis highlighted that increasing dietary full-fat BSF meal inclusion levels caused a drastic decrease of unsaturated fatty acids and carbohydrates in zebrafish brain samples. These results suggest possible compensatory pathways developed by the fish [**116**].

For commercially relevant species, only a few studies that applied FTIR technique are available. Recently, Giorgini et al. [**71**] analysed by FTIR spectroscopy cryosection of medium and hind intestinal tracts of rainbow trout, with emphasis on the mucosa layer. The spectral outcomes were compared to those obtained through the classical histological analysis, based on three different staining methods. The hyperspectral imaging analysis confirmed that the distribution of the most represented macromolecules followed the well-known arrangement of intestine tissues. In addition, the hyperspectral imaging provided a proper outline on the macromolecular composition and the building blocks of rainbow trout intestinal mucosa, through a semi-quantitative information obtained by univariate analysis of the spectral data.

Moreover, the effects of practical diets with increasing full-fat BSF meal levels (0, 25 and 50 % with respect to FM) on the macromolecular composition of liver from rainbow trout juveniles have been investigated [**90**]. Results showed that liver samples from fish fed diet with 50% BSF meal inclusion level (with respect to FM) contained higher amounts of lipids and glycogen compared to a control group.

Finally, FTIR was used to assess the liver macromolecular composition and small intestine nutrient absorption in Siberian surgeon fed diets in which 50% of dietary FM was replaced by full-fat BSF prepupae meal [**91**]. As regards liver, a significant decrease of total lipids, fatty acids and glycogen was observed compared to the control group fed a FM control diet. Conversely, no change was observed in the macromolecular composition of small intestine samples.

Based on these results, FTIR spectroscopy represents a reliable tool for the analysis of different fish organs and tissues, providing at the same a correlation between chemical and morphological features and, as further extent, improving histological outcomes.

Microbiome

Dietary composition is one of the key factors in shaping fish gut microbial communities [**133**,**134**] which in turn can modulate fish metabolism, intestinal mucosa development and maturation, immunity and disease resistance [**135**,**136**].

The use of BSF prepupae meal as aquafeed ingredient has been shown to positively affect fish intestinal microbiota biodiversity, regardless of the insects' life-cycle stage and/ or defatting process, the insects' dietary percentage of inclusion or the fish species analysed [**51**,**106**,**110**,**137**,**138**]. Presently, most of the studies on the effects of BSF-based diets on the microbiome have been performed on rainbow trout [**51**,**99**,**110**,**137**,**139**].

Bruni et al. [**99**] explored the effects of 25 or 50 % of FM replacement with partially defatted BSF prepupae meal in rainbow trout using the denaturing gradient gel electrophoresis (DGGE), highlighting an increase in intestinal bacterial diversity in fish fed BSF-based diets. DGGE is a well-established, reproducible, rapid and less expensive molecular tool based on genetic fingerprinting. DNA or RNA extraction is followed by the amplification of genes encoding the 16s rRNA and then by the analyses of amplification products by a denaturing gradient gel electrophoresis. In addition, the identification of community members is made possible by the sequencing of excised bands or by hybridisation analyses by specific probes [**140**,**141**].

An increased biodiversity was reported also by Józefiak et al. [**110**], through the fluorescent *in situ* hybridisation, including 20% of full-fat BSF prepupae in rainbow trout diet. This hybridisation technique based on nucleic acids allows the identification of microbial species using group or species-

specific fluorescent labelled oligoprobes avoiding the use of DNA extraction and polymerase chain reaction (PCR) [**142**]. These results were in line with further studies performed on rainbow trout [**51**,**137**,**139**] that evidenced an increased microbiome biodiversity with dietary administration of up to 30% of defatted BSF prepupae meal, using high throughput sequencing method (Illumina MiSeq). This next-generation sequencing tool allows to inexpensively produce large volumes of sequence data and to have a higher power of resolution in detecting microbial species compared to conventional methods [**143**,**144**]. Illumina sequencing was also utilised in studies performed on Atlantic salmon [**138**] and Siberian sturgeon [**91**] that, in agreement with the previous studies, highlighted an increase in microbial community diversity in fish fed diets including 15% of partially defatted BSF larvae meal or 50% of full-fat BSF prepupae meal, respectively.

A higher microbiome diversity is generally considered as an indicator of improved gut health, while a reduced diversity has frequently been associated to gastrointestinal tract colonisation by pathogens [**145**,**146**]. The overall increased diversity and the positive modulation of intestinal microbial communities in response to different levels of dietary BSF meal inclusions have been mainly attributed to chitin. Chitin is generally considered not easily digestible by fish but represents one of the main growth substrates of lactic acid bacteria (LAB) [**147**,**148**]. LAB are related to *Firmicutes* and *Actinobacteria* phyla which usually represent the 'core gut microbiota' in different marine and freshwater species [**149-153**]. These bacteria, using chitin as prebiotic, are crucial in making available indigestible carbohydrates leading to a better nutrient accessibility and utilisation for fish [**154**]. In addition, LAB contribute to the synthesis of vitamins and short-chain FA, considered as the primary enterocyte's energy source [**149**], or of important anti-inflammatory molecules, like butyrate [**155**,**156**].

Firmicutes and *Actinobacteria* phyla have been shown to increase in intestinal mucosa and digesta of rainbow trout fed with defatted BSF prepupae meal (up to 50% of inclusion) [**51**,**99**,**139**] or full-fat BSF prepupae and larvae meal (30% of inclusion) [**137**]. In particular, Terova et al. [**139**] reported that the dietary defatted BSF inclusion increased the relative abundance of *Lactobacillales*, mainly represented by *Lactobacillaceae* and *Leuconostocaceae*, involved in starch and fibres digestion as well as of bacteria from the order *Clostridiales* that includes many butyrate producers like *Clostridium butyricum.* Similarly, an enhanced colonisation of *Lactobacillus* sp. was observed in rainbow trout fed a diet including 20% of full-fat BSF prepupae meal [**110**] and in Siberian sturgeon fed 15% of full-fat BSF larvae meal [**106**]. As reported by Huyben et al. [**137**], the abundance of *Bacillaceae* (phylum *Firmicutes*) was higher in rainbow trout fed BSF-based diets and this increase was attributed to the high level of dietary chitin since these bacteria are able to use chitin through the endogenous production of chitinase. Furthermore, the same authors demonstrated that the presence

of *Corynebacterium* (phylum *Actinobacteria*) was higher in rainbow trout fed the full-fat BSF larvae or prepupae meal with respect to the defatted larvae meal. This underlies the ability of *Corynebacterium* to use dietary lipid through the activation of endogenous lipases [**157**]. An increased abundance of *Corynebacterium* was also observed in Atlantic salmon fed on partially defatted BSF-based diet (15% of inclusion) with respect to the control diet [**138**]. Furthermore, chitin and its deacetylate derivate chitosan have antimicrobial properties and bacteriostatic effects, particularly on some Gram-negative pathogen bacteria [**158-160**]. This defence mechanism could be enhanced by LAB which are able to produce bactericidal compounds like lactic acid, hydrogen peroxide and biosurfactants preventing the adhesion to intestinal mucosa of pathogens like *Staphylococcus aureus*, *Streptococcus agalactiae* and *Pseudomonas aeruginosa* [**161**,**162**].

Several studies on rainbow trout fed defatted BSF prepupae meal diets (up to 50% of inclusion) [**51**,**99**,**139**] or full-fat BSF prepupae and larvae meal diets (30% of inclusion) [**137**] revealed a reduction in both gut digesta and mucosal-adhered *Proteobacteria*, a Gram-negative phylum containing pathogens. In particular, Rimoldi et al. [**51**], reported that 20 or 30 % of defatted BSF prepupae meal dietary inclusion increased the number of bacteria belonging to *Mycoplasma* genus which is associated to a beneficial effect on host health due to the production of lactic and acetic acids as main metabolites [**163**]. Similarly, rainbow trout fed 25 or 50 % of defatted BSF prepupae meal inclusion in diets were particularly rich in *Pseudomonas stutzeri* [**99**] which possess antiviral activity and was listed as a probiotic bacterium [**164**,**165**]. In contrast, in rainbow trout, a 20% full-fat BSF prepupae meal inclusion caused an increase in the *Clostridium coccoides* abundance [**110**] which is known to play an important role in fish immune response and pathological processes [**166**].

Finally, recent studies performed on zebrafish fed diets with increasing inclusion levels of full-fat BSF prepupae (0, 25, 50, 75 or 100 %) showed contradictory results. In zebrafish larvae, the positive effects of dietary BSF inclusion was highlighted by the highest microbial community biodiversity showed by the group fed 50% substitution of FM with full-fat BSF prepupae meal as well as by the *Vibrio* abundance which was negatively influenced by the dietary increasing inclusion of full-fat prepupae meal [**33**]. Conversely, the same BSF dietary inclusion levels caused in zebrafish juveniles, a reduction of microbial diversity [**116**]. However, the overall fish gut health was preserved since BSF prepupae meal is particularly rich in lauric acid which is known to possess anti-inflammatory and antimicrobial properties against Gram-positive bacteria [**167-169**].

It should be pointed out that to fully unveil the response of intestinal microbiota to BSF dietary inclusion levels, differences between mucosa-adhered and gut digesta communities must be considered since some allochthonous species could poorly colonise host intestine with a lower biodiversity with respect to the intestinal content [**170**]. Different studies on BSF prepupae meal

administration in salmonids reported that microbial diversity was higher in gut digesta than in intestinal mucosa [**99**,**138**]. For that reason, analysing only gut digesta- or intestinal mucosa-associated microbiota or a mixture of both could partially underestimate microbial community response to dietary changes, while it is recommended to profile mucosal and digesta communities separately when feasible.

Molecular biology (real-time PCR): growth factors, immunity, stress, chitinases, lipid metabolism, and appetite stimulus

One of the most robust and widely used methods for gene expression quantification is the real-time PCR which is able to distinguish and amplify a specific nucleic acid sequence in a sample, monitoring the amplification process through the correlation between product concentration and fluorescent intensity [**171-173**]. This technique has been used in some of the most important finfish species like gilthead sea bream [**174**], European sea bass [**175**], Atlantic salmon [**176**], Siberian sturgeon [**177**], sole [**64**], ballan wrasse [**178**] and rainbow trout [**179**]. However, because of the lack of complete and easy genomic information availability about these last species its application is still limited [**180**].

With the use of BSF meal in aquafeed formulation, studies about the application of real-time PCR over the last few years are quite limited and mainly focused on growth factors, fish immune and stress response, appetite stimulus, lipid metabolism and chitinolytic activity.

Considering growth factors [insulin-like growth factors (*igf1*, *igf2a*) and myostatin (*mstn*)], BSF prepupae meal dietary inclusions usually resulted in good growth performance in most of the studies. In both zebrafish larvae and in rainbow trout juveniles, an increase in growth parameters was well supported by the hepatic gene expression of the growth factors [**33**,**90**]. These results support the hypothesis that BSF prepupae meal possesses a proper amount of proteins (up to 60% dry weight) and a well-balanced amino acids (AA) profile (similar to that of FM) which are key dietary features to guarantee a proper aquaculture production [**34**].

Fish growth is strictly related to feed intake and the brain (particularly the hypothalamus) is a key-actor in regulation of energy metabolism, nutrient absorption, and the control of feeding activity. However, it should be considered that the gastrointestinal tract is connected to the brain in metabolic and appetite control. The gut-brain crosstalk occurs through the release of a number of gut peptides that exert responses within the brain, as well as through neuroendocrine and sensory inputs from the gut [**181**]. Other signals are also involved such as nutrient levels, through central nutrient sensing systems, and the presence/absence of food in the gastrointestinal tract, through vagal afferents projecting to the brain [**182**]. To date, studying the gene expression of both orexigenic [ghrelin (*ghrl*), neuropeptide y (*npy*) and cannabinoid receptor 1 (*cnr1* or *cb1*)] and anorexigenic [leptin (*lepa*) and

melanocortin 4 receptor (*mc4r*)] signals represents a valid tool to better understand fish responses to new formulated diets. No significant variation in *ghrl* gene expression in medium and distal intestine of rainbow trout juveniles fed with practical diets containing 30 or 60 % substitution levels of vegetable proteins with BSF prepupae meal was recently evidenced [**111**]. However, in the same experimental group, but considering brain, appetite signals analysed (*cb1*, *npy* and *mc4r*) showed a significant downregulation [**111**]. Differently, in zebrafish larvae and juveniles, gene expression of orexigenic signals (*ghrl*, *npy* and *cb1* analysed in whole fish samples for zebrafish larvae; *ghrl* and *cb1* in intestine samples for zebrafish juveniles) increased with the increasing dietary BSF prepupae meal inclusion levels [**33**,**116**] fully supporting growth.

While BSF meal has been proven to be an adequate protein source, its inclusion in aquafeeds is still possibly limited by the chitin content [**44**,**183**] and its (often) unbalanced fatty acids profile [**47**].

Over the last years a controversial role has been attributed to chitin in aquafeed formulation [**10**]. High dietary inclusion levels of BFS meal (and possibly of chitin) often induced a fish growth reduction [**91**,**103**,**105**]; however, no direct correlation between chitin feed content and fish growth reduction has yet been demonstrated. Chitin can also have a beneficial activity on the fish immune system, lower stress response and improved gut health [**21**,**110**,**148**].

The use of molecular markers represents a valid and up to date tool since several biomarkers are available, for different fish species, to assess stress and immune response. Studies demonstrated the correlation between increased gene expression levels of glucocorticoid receptor and heat shock proteins and exposure to stressors (including malnutrition) [**64**]. Pro-inflammatory cytokines such as interleukin 1-beta (*il1b*), tumour necrosis factor a (*tnfa*), anti-inflammatory cytokines like interleukin-10 (*il10*), and inflammation mediators such as nuclear factor kappa-light-chain-enhancer of activated B cells (*nfkb*) and myeloid differentiation primary response 88 (*myd88*) represent useful markers of inflammation, able to provide early information when testing new ingredients in aquafeed formulation [**184-186**]. However, the presently available results are still controversial and fish responses seem to be species and stage specific as well as related to BSF meal dietary inclusion levels.

Gut gene expression of immune response markers in adult zebrafish (*il1b*, *il6* and *tnfa*) [**103**], and pre-smolt (*il4*, *tgfβ1*, *il10*, *ifnγ*, *il8* and *myd88*) [**50**] and seawater-phase (*il1b*, *il17a*, *myd88*, *il8*, *il4*, *mhcl*, *il10*, *ifnγ*, *tgfβ1*, *cd8β*, *cd3γδ* and *foxp3*) [**100**] Atlantic salmon was not negatively affected by the BSF-meal dietary inclusion level up to 50, 60 or 100 %, respectively. Conversely, a dietary full-fat BSF inclusion of 50% or higher resulted in the immune response activation in both larval and juvenile zebrafish intestine (*il1b*, *il10*, *il6* and *tnfa*) [**33**,**116**,**126**] and in medium intestine of rainbow trout juveniles (*il10*, *tnfα* and *tlr-5*) [**90**] as well as in the stimulation of regulatory T cell activity in the proximal and distal intestine of Atlantic salmon (*cd3γδ* and *foxp3*) [**50**].

Inflammatory events detected by real-time PCR can be coupled with a higher stress response: an increase in the hepatic *hsp70.1* gene expression was detected in rainbow trout juveniles [**90**] and pre-smolt Atlantic salmon [**50**]. Accordingly, in zebrafish larvae and juveniles, an increased dietary full-fat BSF prepupae meal inclusion resulted in a higher hepatic *hsp70.1* and glucocorticoid receptor (*nr3c1*) gene expression [**33**,**116**]. However, in Atlantic salmon fed diets with higher full-fat BSF meal inclusion (85% of the diet protein content) the hepatic expression of *hsp70.1* was not affected by the diet [**176**].

Finally, due to the unbalanced fatty acid profile of BSF meal, with emphasis on their lack of PUFA, a number of biomarkers related to long-chain PUFA biosynthesis and fatty acids metabolism is now available and represents a great opportunity for better understanding the biosynthetic pathways in different fish species in response to dietary treatments. Most of the studies so far published focused their attention on elongase and desaturase gene expression. Specifically, Bruni et al. [**118**] demonstrated that the *elovl2* and *fads2* gene expression in the pyloric caeca of rainbow trout juveniles tended to increase with the increasing amount of the dietary full-fat BSF meal inclusion (25 and 50 %). Accordingly, in both zebrafish larvae [**33**] and adults [**103**,**132**], an upregulation of *elovl2*, *elovl5* and *fads* hepatic gene expression was observed in experimental groups fed the highest full-fat dietary BSF prepupae meal inclusion (100 and 50 %, respectively). The dietary lack of PUFA in freshwater fish fed BSF-based diets could enhance the conversion of shorter-chain fatty acids in highly unsaturated ones through the activation of elongation and desaturation pathways [**65**]. Furthermore, an increasing expression of markers involved in fatty acids (*cd36*, *fabp2*) and cholesterol (*npc1l1*) uptake has been observed in the proximal intestine of Atlantic salmon fed diets including 60% of full-fat BSF meal [**50**].

While real time PCR may represent a valid tool for the early detection of many physiological changes in fish, if applied to nutritional studies its employment should be coupled with other laboratory techniques, like histology and infrared spectroscopy, to obtain a clearer overview. Further research is necessary to overcome the lack of complete and easily available genomic sequences for farmed fish species and to make real-time PCR a ‘routine technique’ in nutritional studies.

Fish quality

The consumers’ perception process of food quality has been summarised by Fernqvist and Ekelund [**187**]. The authors showed how it is influenced by intrinsic quality attributes (*i.e.*, sensory properties) and intrinsic and extrinsic quality cues which compose quality expectations. Among the firsts, attributes perceived before the consumption like colour, size, and damages are listed. The latter aspects refer to label, packaging, and other external factors. In addition to this, consumers are

increasingly focusing on health, eco-friendliness, sustainability of food [**187**] suggesting that people are looking for deeper sensory pleasures from foods, moving from the sensory properties to health and ethical motivations [**188**]. On this regard, the aspects related to animal farming, such as animal right, welfare, and feed assurance, are embedded into the 'new' concept of meat quality [**189**].

To date, researchers have useful tools to objectify many of the quality parameters, such as physical properties, chemical composition, sensory properties, and consumers' liking which have been applied to answer if BSF meal inclusion in fish diets may affect fish quality and consumers' acceptance. Physical attributes, such as fillet colour, water holding capacity (WHC) and texture are commonly evaluated by instrumental methods. For instance, colorimeters have been utilised by different authors while performing a colour evaluation of fillets from Atlantic salmon [**190**] and rainbow trout [**102**,**118**,**191**] fed diets with different inclusion levels of partially or not defatted BSF larvae or prepupae meals. This instrument is designed to assess the colour of a sample in a user-friendly way (easy to use and fast response); however, the punctual reading of the colorimeter requires at least two- or three-points determinations in the same sample. Mancini et al. [**192**] instead, captured the whole fillet image through a digital camera, then the colorimetric measurement was carried out by ImageJ software. Irrespective to the instrument utilised, results are generally presented using the CIELab colour space [**193**], thus splitting the colour into three axes explaining the lightness (L*), redness (a*), and yellowness (b*) values of the sample. In this way, colour perception and its variations are difficult to interpret. Indeed, data showed as L*, a*, and b* values do not allow an inexpert reader to understand the overall sample colour perception. As a consequence, the differences observed through these methods among the samples did not answer the question: 'were the differences among colour of the samples perceived by an observer?'. A useful tool to distinguish different colours is the Delta E (ΔE) which is defined as a difference in sensation, as revealed by the name itself: Delta is a Greek letter that stands for the incremental change of a variable, while the 'E' is the abbreviation for 'Empfindung', the German word for sensation. As a whole, ΔE shows the distance between two colours and it is calculated as proposed by Sharma and Bala [**194**]. Furthermore, Mokrzycki and Tatol [**195**] reported different ΔE ranges based on the observer perception of the colour difference, as follows: $0<\Delta E<1$ - observer does not notice the difference; $1<\Delta E<2$ - only experienced observer can notice the difference; $2<\Delta E<3.5$ - inexperienced observer also notices the difference; $3.5<\Delta E<5$ - clear difference in colour is noticed; $\Delta E>5$ - observer notices two different colours. While looking at the results showed in the literature, it seems that the dietary treatments with BSF did not affect colour values of the rainbow trout fillets [**102**], with the exception of yellowness which was, in some cases lowered [**118**,**192**]. In the case of Mancini et al. [**192**], where ΔE was proposed, control (C) and fillet from trout fed BSF at 25% of substitution (HI25) showed the same perceived colour (ΔE value =

0.39), whereas the ΔE values calculated between fillets from C and trout fed BSF at 50% of substitution (HI50) or between HI25 and HI50 fillets were 2.96 and 2.60, respectively, thus being perceived as different. The inconsistent results found until now can be due to the complex interactions composing the overall sample colour. For instance, the flesh colour may be directly affected by the pigments contained in the administered diets (i.e. carotenoids), especially carried by vegetable ingredients (soybean, corn) and BSF meal, in a minor extent [**191**]. The addition of astaxanthin in the diet for salmonids, inferable from the high a* values reported for Atlantic salmon [**190**] and rainbow trout [**192**] fillets, could drown out the effect of dietary ingredients on the different indexes. In addition, lipid oxidation might produce yellow pigments, hence high b* values can be found in fillets with high malondialdehyde equivalents content (MDA-eq.), as in the case of rainbow trout fillets fed on BSF meal-free diet (b*: 5.57; MDA-eq.: 0.55 mg/kg fillet) or containing 25% of full-fat BSF prepupae meal (b*: 4.21; MDA-eq.: 0.30 mg/kg fillet) [**118**].

Considering WHC and texture, these two physical properties are strictly connected, since the WHC (defined as the ability of a food to retain its own water after the application of a pressure, centrifugation, or heating) has proved to play a key role in the formation of food texture [**196**]. The most common method adopted for WHC assessment is the one proposed by Hultmann and Rustad [**197**] and modified by Iaconisi et al. [**198**] based on a centrifugation at 510×*g* for 5 min. of a 2 g sample with known water content. Applying this method to fillets from BSF meal fed fish, Bruni et al. [**118**,**190**] did not find any significant difference nor among rainbow trout or Atlantic salmon samples. Similarly, Secci et al. [**191**] did not show any significant WHC modifications of rainbow trout fillets as affected by BSF meal inclusion in fish diet, either after frozen storage (120 days at -10 °C) and cooking (boiling at 95-98 °C for 5 min). An indirect measurement of WHC is obtained by calculating the cooking loss as the weight difference of the fillet before and after the cooking process, according to the formula: 100 × [(raw fillet weight - cooked fillet weight) / raw fillet weight]. Consistently with the WHC assessment, both Borgogno et al. [**199**] and Secci et al. [**191**] did not underline significant dietary effects on this parameter. Texturometer is generally utilised for texture measurements. The instrument can be equipped with a variety of load cells (as 1 kN or less) and probe (cylindrical, straight blade, Warner-Bratzler) based on the type of test the users want (shear test, texture profile analysis, etc.). Other parameters, such as cross-head speed and the percentage of total deformation have to be set. For this reason, results obtained with different methods and by different researchers are not comparable. Despite this, the textural properties of the fillets from fish fed diets containing BSF meal as alternative protein source seemed not to vary with respect to a control diet, irrespective to the substitution levels (commonly 25, 50, 75 % of FM replaced) and the investigated species, rainbow trout [**191**,**112**] and Atlantic salmon [**190**]. Analogous outputs were obtained while

using *Tenebrio molitor* larvae meal as protein source at 25 or 50 % of inclusion for feeding blackspot sea bream, *Pagellus bogaraveo*, [**198**] or rainbow trout [**200**]. In conclusion, from the instrumentally evaluation of the physical properties of fish fillets emerged that BSF did not impair these items even if colour modification needs to be considered when formulating the experimental diets.

Fish are an important source of energy, essential and non-essential AA, minerals (iodine, selenium, calcium) and vitamins (A, D) and a functional food, being one of the main sources of the long-chain PUFA, as eicosapentaenoic and docosahexaenoic acids (EPA and DHA, respectively). A weekly consumption of around 1.75 g (for adults) and 3.5 g (during pregnancy and lactation) of EPA+DHA is highly recommended while following a healthy diet [**201**], since they play positive roles in the prevention of body overweight and obesity [**202**] and in the protection against cardiovascular diseases [**203**]. Hence, fish quality needs to be evaluated considering flesh chemical composition, by means at least of the AA and fatty acids profiles. The methods utilised for these analyses were well established and no significant implementations have occurred in the recent published articles [**118**,**190**,**199**,**204**,**205**]. Specifically, total AA composition is commonly evaluated using HCl to hydrolyse fish samples prior to assess a liquid chromatography, as ultra-performance or high pressure ones. Results are commonly expressed as mg AA/g muscle. Concerning fatty acids, once total lipids are extracted from the flesh (methanol:chloroform 2:1 v/v being the most utilised solution) and quantified (gravimetrically), they are subdued to a saponification, then they are methylated to obtain fatty acids methyl esters (FAME). An internal standard as 19:0 [**35**,**204**] or 23:0 [**118**,**190**,**191**,**199**] is added to the samples. Finally, fatty acids composition is determined using a gas-chromatograph (GC) coupled with a flame ionisation detector. The comparison between the recorded chromatograms with the one of a pure standard FAME mix allows the researchers to qualitatively determine the fatty acids profile, thus the results are expressed as % of total FAME. A quantitative analysis is also possible when using a calibration curve obtained with the FAME standard mix and it is highly suggested. Indeed, the fatty acids content (g/100 g muscle) is necessary to retrieve the fundamental information on the EPA+DHA level in the sample, hence, to know if the samples make a valuable contribution to a balanced and healthy diet. Nevertheless, the fatty acids content of fillet from fish fed BSF meal is still scarcely depicted [**35**,**102**,**118**,**191**]. However, as showed by several studies, fillet fatty acids profile represents the main weakness of BSF meal inclusion in fish diet, while the total AA both of Atlantic salmon muscle [**35**,**204**] and rainbow trout plasma [**46**] were scarcely affected. Since the early work of St-Hilaire et al. [**49**], it was evident that the partial substitution of FM (or of the overall protein content) with BSF meal dramatically increased rainbow trout fillet SFA while decreasing the PUFA fraction. Numerous subsequent studies have confirmed this finding irrespective the fish species investigated [**35**,**46**,**102**,**103**,**118**,**190-192**,**199**,**204**,**205**]. Since BSF meal

is rich in lauric acid (12:0), fillet increase in SFA was expected in fish fed BSF diet. In addition, while adding BSF meal to the diet, a reduction of FO, which is the main source of n3 PUFA, could be necessary to obtain iso-lipidic and iso-energetic diets. The sum of these elements leads PUFA depletion in fish flesh which, in turn, lowers the overall nutritional quality of the fillet. For this reason, mitigation actions were investigated such as the use of partially defatted BSF meal [**102**], the use of BSF grown on substrates rich in n3 PUFA [**32**,**49**], or the avoidance of FO reduction in feeds [**190**]. However, interesting information comes from the recent research of Bruni et al. [**118**] who observed attenuated fatty acids profile differences in rainbow trout fed full-fat BSF prepupae meal (inclusion levels set at 0, 25 or 50 %) relative to the dietary differences. Surprisingly, the n3 PUFA as well as EPA and DHA amounts were not impaired by the dietary intervention, thus resulting in a well-balanced fatty acids profile of the fillets. Standing on our knowledge, Bruni et al. [**118**] linked for the first-time fillet quality and fish lipid metabolism, finding that the formulated practical diets containing full fat BSF meal were effective in increasing pyloric caeca *elovl2* and *fads2 gene* expression which in turn positively modified the fillet dietary fatty acids profile. This topic deserves further investigations.

The modifications occurring both on lipid and protein fractions while handling fish fillets (*i.e.* storage and cooking) promote the development of the fish volatile profile (referred as volatile organic components; VOCs). Recently, Nieva-Echevarría et al. [**206**] found significant different volatiles comparing wild and farmed European sea bass which the authors associated to contaminants and diets. In addition, an interaction between fish growing conditions and cooking methods was highlighted by the same authors, because of a different lipid content and fatty acids profile. Hence, a possible effect of BSF meal inclusion in the diet on farmed fish can be also hypothesised. The most common method to evaluate VOCs composition is based on a solid-phase extraction, followed by the GC-mass spectrometry (MS) analysis of the sample headspace, as described in Iglesias and Medina [**207**] and modified by Fratini et al. [**208**]. For this purpose, the extracted samples were inserted in a vial and heated (60 °C for 30 min.) to collect VOCs in the headspace of the vial. The VOCs were absorbed on a fibre, as Carboxen™/Polydimethylsiloxane (75 μm) [**192**] prior to be GC-analysed. The identification of components is generally conducted by consulting available libraries and comparing with mass spectra and retention times of commercial standards. At the best of authors' knowledge, the articles showing the VOCs content are limited in literature. Among these, the paper by Mancini et al. [**192**] offers interesting results while assessing VOCs profile of BSF larvae meal, rainbow trout feeds (control, 25 or 50 % of FM substitution with partially defatted BSF larvae meal), and fish fillets. Although the dietary VOCs profile was effectively different, Mancini et al. [**192**] showed that the VOCs profile of fillets was scarcely affected by the presence of BSF in the diet for

rainbow trout, irrespective the substitution level. Such result could be attributable both to a molecule degradation, occurring during the digestive process of the fish, and/or to an absent muscle deposition. An innovative approach was adopted by Bruni et al. [**190**] while evaluating the volatile composition of raw Atlantic salmon fed diets containing 25 to 75 %. BSF meal larvae. The proton transfer reaction-time of flight-mass spectrometer (PTR-ToF-MS) technique offers technical advantages as a rapid data collection (100 s for each sample) and the possibility to evaluate VOCs of the whole sample at room temperature, hence avoiding sample manipulation (mincing, solid phase extraction), and heating. Despite its pros, PTR-ToF-MS technique requires numerous and time-consuming offline activities, corresponding to the spectra-calibration, raw data acquisition and correction (elimination of peaks imputable to water chemistry, interfering ions, and also elimination of all peaks for which the average concentrations are lower than an established threshold), and tentative peak identification based on literature or libraries. This last step appears to be critical, since 18 out of 29 VOCs were quantified without being identified, as depicted in Bruni et al. [**190**] results. Concerning the effect of BSF meal on Atlantic salmon, data agree with Mancini et al. [**192**] since no different VOCs profiles emerged among the samples.

In conclusion, the inclusion of BSF meal up to 25% did not jeopardise fillet physical and nutritional characteristics, while upper inclusion levels mainly affect colour and fatty acids composition. Despite this, understanding if and how the diet affects fillet sensory properties and consumers' liking is necessary to positively judge BSF meal as protein source for aquafeeds. The early study by Sealey et al. [**46**] approached the topic through a sensory evaluation of rainbow trout fillets conducted by 30 untrained panellists (14 males and 16 females, from 18 to 65 years old). The method adopted was a triangle difference test, which consists of indicate the odd sample in a set of three blinded samples. Panellists were also free to express the reason of their choice and what were the attributes making different the sample perception. Sealey et al. [**46**] showed that people were unable to discriminate between fillets from rainbow trout fed a control diet (containing anchovy meal) and the experimental diets containing normal and enriched (with n3 PUFA) BSF prepupae meal. Recently, the intensity of the perceived sensory attributes of fish fed BSF meal was evaluated by using trained [**199**,**204**] or untrained assessors [**205**]. For instance, Stadtlander et al. [**205**] proposed an organoleptic test on steam-cooked fillets from rainbow trout fed a diet where 46% of FM was substituted by BSF meal. Fifteen untrained panellists were asked to rate different sensory characteristics (odour, colour, texture and taste) on a scale between 0 (does not apply) to 9 (applies fully). Similarly, Belghit et al. [**35**] tested raw and baked fillets from Atlantic salmon fed diets at increasing substitution level of FM with BSF meal. Ten trained assessors were asked to evaluate the intensity of several sensory attributes (odour, colour, texture) by using a 15-cm non-structured continuous scale. Results from the previous

mentioned studies mostly agreed. Indeed, the sole significant variation detected was a difference in fillet colour even if Stadtlander et al. [**205**] found darker flesh in fillets from trout fed insect meal, while the salmon fed 66% of BSF meal had fillets significantly less coloured than those of the group fed the control diet (without BSF) in Belghit's et al. [**204**]. An innovative approach distinguishes the work of Borgogno et al. [**199**], who found that BSF larvae meal inclusion at 20 and 40 % in rainbow trout diets affected the fillet sensory profile when assessed through a descriptive analysis and a temporal dominance of sensation (TDS) methods by trained panellists. Several outcomes can be retrieved from the proposed methodology, both in terms of sensorial attributes related to aroma, texture, appearance, and tactile sensations and of the sensory perception process during all the chewing. The main criticism of these methods is about the training sessions. Indeed, both descriptive analysis and TDS require three training sessions of about 60 min. each, that means overall 6 sessions dedicated for training more the time necessary for sensory evaluation. Consumers' expectation and willingness to pay were investigated by Ferrer Llagostera et al. [**53**] while Bruni et al. [**190**] focused on consumers' liking. On one hand, discrete choice experiment (DCE) was chosen by Ferrer Llagostera et al. [**53**] to understand the preferences of Spanish people towards farmed gilthead sea bream fed diets containing insect meal. DCE is basically an on-line questionnaire which proposes different products (in this case gilthead sea bream fed insect meal) at different price levels in several purchase situations. In the case of Ferrer Llagostera et al. [**53**], the purpose was to identify the consumers' trade-offs in their choice decision. On the other hand, consumers' test was selected by Bruni et al. [**190**] to investigate Italian's liking and intention of re-consumption of Atlantic salmon obtained by administering BSF meal to fish as protein source in aquafeed. The authors served Atlantic salmon blinded samples at 80 consumers, asking them to express their like or dislike (using a 9-points scale) for a series of sensory attributes. Interestingly, Ferrer Llagostera et al. [**35**] highlighted that people had a good opinion of gilthead sea bream farmed using insects as feed ingredients, but they expected these fish to taste 'less'. On the contrary, the hedonic evaluation proposed by Bruni et al. [**190**], conducted under no informed condition (consumers did not know what was the dietary treatment), gave important information. Firstly, people perceived as too pale the colour of fillets from groups fed diets with 66 and 100 % BSF meal inclusion levels and the flesh resulted slightly firmer than the control group (0% BSF meal), in line with the instrumental analyses [**204**]. Moreover, Bruni et al. [**190**] point out that increasing substitution level of BSF meal (0, 33, 66 and 100 %) in Atlantic salmon diet did not counteract consumers' overall liking and intention of re-consumption (>70% of positive answers). The results from Spanish and Italian research groups give reason to hope for a complete consumers' acceptance of insect meal as protein source in the aquaculture sector.

Conclusions and future perspectives

Several analytical methods and laboratory techniques are presently available to deepen our knowledge about fish welfare and quality in response to diets including BSF meal. While these laboratory techniques have been used in fish and other animal's responses to all kind of alternative ingredients, a number of studies have recently showed their suitability for a better understanding of fish responses to BSF based diets. Results evidenced that certain dietary inclusion levels of BSF meal are able to promote fish health, welfare and quality of the product. However, there is a lack of information about long-term use of these diets. For this reason, scientists should perform further studies over longer periods of time and possibly over the whole fish life cycle. On this regard, emphasis should be given to nutritional programming experiments as well as the possible effect of these new diets on fish reproduction. The laboratory methodological approaches included in this review article may serve as a starting point for this further research. Considering these last aspects, the authors suggest a constructive crosstalk between research and industry to sustain the development of a high quality and sustainable aquaculture.

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Chapter 6

Conclusion

The present PhD thesis aimed to evaluate the possible transition to future feeds in aquaculture characterized by lower amounts of conventional marine and plant -derived raw materials by using BSF prepupae meal as a more sustainable ingredient for aquafeed formulation. In particular, this insect species was selected due to its promising protein content and amino acidic profile, the environmental-friendly rearing and the possibility to take advantage of its high feed conversion efficiency to ameliorate its nutritional profile (especially in terms of n3 PUFA) and to apply the circular economy concept to aquaculture sector.

Due to the lack of information about long-term use of BSF-based diets in fish nutrition, the first trials were mainly focused to test the effects of full-fat BSF prepupae meal inclusion respect to FM (25 and 50 %) over the whole life cycle of zebrafish. Both experimental diets did not show major negative effects on zebrafish growth and gut health, confirming the beneficial role which bioactive molecules like chitin and lauric acid, largely contained in BSF prepupae meal, can exert on fish gastrointestinal tract. However, the BSF prepupae meal unbalanced fatty acids profile was able to impair liver health, causing hepatic steatosis, and reproductive performances of female zebrafish when the higher inclusion level respect to FM was used (50%). In particular, a general decrease in spawned eggs was evident, possibly due to a delay in oocyte maturation caused by an inadequate dietary intake of n3 PUFA. However, the hatching success was not negatively affected highlighting that females favoured gametes quality rather than quantity.

To address this critical aspect of the BSF prepupae meal nutritional profile and to introduce circularity in the aquaculture sector, the second section of the present PhD thesis aimed to test new BSF-based diets (characterized by 25, 50, 75 and 100 % of inclusion respect to FM) formulated using BSF prepupae reared on coffee industry by-products (coffee silverskin) enriched with 10% of *Schizochytrium* sp. as a source of PUFA. Besides the recycling of coffee silverskin, the waste product of coffee roasting, this enrichment procedure represented a valid method to improve the insects' final biomass in terms of PUFA providing the possibility to extend up to 50% the well-tolerated percentage of BSF prepupae meal dietary inclusion respect to FM. In fact, in zebrafish larval development and over a long-term feeding trial on juveniles and adults, the 50% BSF prepupae meal dietary inclusion level respect to FM did not show major negative effects on fish overall health, thus representing the best compromise between ingredient sustainability and proper fish growth and welfare. Higher inclusion levels respect to FM (75 and 100 %) caused a stress response in fish leading to a reduction of fish welfare, high lipid accumulation in the liver (steatosis) and a strong reduction of PUFA content of fish fillets. Furthermore, these high inclusion levels deeply affected female zebrafish reproductive performances by reducing the spawning rate and the hatching success.

However, the nutritional programming through parental feeding enhanced the ability of the first filial generation (F1) to utilize the same BSF-based diets provided to adults during their whole life-cycle. Interestingly, growth, hepatic lipid accumulation and gut and liver health were not impaired when F1 zebrafish larvae were fed diets characterized by up 100% of FM substitution level with BSF prepupae meal. Nutritional programming should thus be considered as one of the potential solutions for counteracting the recurring negative side effects of high BSF prepupae meal dietary inclusion levels. However, further studies are necessary to understand the long-term effects of BSF-based diets on later life cycle stages of F1 and, possibly, on further generations.

In all the above-mentioned studies, the increase of SFA and the parallel decrease of PUFA in the diets used and, thus, the negative effects on zebrafish physiology has been only ascribed to the unbalanced fatty acids profile of the BSF prepupae meal used. However, it should be pointed out that in the studies of both first and second section, diets with a progressive decrease of FO were used. This strongly suggests the importance of this ingredient as a source of HUFA, both for ensuring fish proper growth and welfare and for human wellbeing. On this regard, the reduced FO availability represents the main current challenge of aquaculture sector. In fact, the worldwide demand of n3 PUFA from seafood is increasing and thus alternative sources and sustainable processes are needed to face the limited supply of these essential fatty acids from fish origin.

The third section aimed to test innovative diets on a freshwater (Siberian sturgeon, *Acipenser baerii*) and a marine (gilthead seabream, *Sparus aurata*) farmed fish species. In the first experiment, the best performing BSF-based diet from zebrafish trial was selected for a feeding trial with Siberian sturgeon juveniles in an aquaponics system, representing an additional important step in light of a future zero-waste aquaculture development. The aquaponic systems performed optimally during the trial demonstrating its feasibility for testing new aquafeed ingredients on farmed fish species, but fish fed BSF-based diet showed lower diet acceptance that resulted in growth and survival reduction, a hepatic lipids and glycogen storage mobilization, and a worsening in gut and liver health.

The second experiment aimed to assess the potential role of BSF prepupae as partial replacer of vegetable protein (singly or in combination with PBM) in practical diet deprived of fish meal intended for gilthead seabream. The dietary inclusion of BSF prepupae meal and/or PBM respect to dietary vegetable proteins resulted in a lower incidence of intestine histological alterations and inflammatory responses. In particular, BSF prepupae meal confirmed also in this fish species the ameliorative effects on gut health, while PBM exerted beneficial effects on intestine nutrient absorption. These alternative ingredients can be thus considered promising alternatives also for farmed fish species, since they can counteract the negative side effects due to the high amount of plant ingredients (used

in their turn as FM replacers) in diets for carnivorous species, like gilthead seabream, promoting healthy aquaculture able to sustain both production and welfare of fish.

Overall, results obtained evidenced that inclusion levels of BSF prepupae meal respect to marine- and plant-derived ingredients up to 50 and 40 %, respectively, can promote fish health, welfare and quality of the product. Specifically, the fish trials performed for the present PhD thesis represent an example of how aquaculture could be implemented with the re-use of land-produced by-products (coffee silverskin as BSF prepupae growth substrate and poultry by-products as aquafeed ingredients) and with the partial substitution of conventional ingredients with more sustainable and eco-friendly ones like BSF prepupae meal, while sustaining the production of healthy fish and reducing the associated environmental footprint. Aquafeeds still represent the 50-70 % of the costs sustained by fish facilities. For that reason, further research is needed to improve the overall knowledge on insect meal, a cost-effective and sustainable ingredient able to promote the further expansion, sustainability, and competitiveness of the aquaculture sector, also encouraging the circular economy between land and aquatic sectors. In light of this, further studies are needed to maximise the use of BSF prepupae meal, still exploiting its main advantages represented by the proper protein content and the presence of important bioactive molecules, while solving the fatty acids-related issue. In this context, enrichment procedures of insects should be further investigated as well as the possible exploitation of the insect lipid fraction, derived from defatting procedures, to obtain long-chain PUFA starting from fatty acids of scarce interest for fish diets production.

Furthermore, in the last years, scientific community has point great attention on the absolute need to evaluate the overall fish welfare when new aquafeed ingredients are tested. The development of several innovative laboratory analyses that can be coupled with more classical approaches allows to obtain a more comprehensive view on the physiological effects of new dietary formulations in aquaculture. Zootechnic indexes, histological analyses, infrared spectroscopy, real-time PCR, microbiome sequencing and biochemical analyses of the fish edible portions are available techniques that can be applied to assess fish growth and welfare. Through the multidisciplinary approach applied in every trial and the exhaustive review on the results obtained in other important farmed fish species, the present PhD thesis may provide a better understanding of fish metabolism and physiological responses to BSF-based diets and provide new scientific knowledge and novel competitive feeding formulations to be transferred to feed industries, farmers and stakeholders promoting further aquaculture development.

Chapter 7

Appendix

7.1

Dissemination of results and awards

Scientific publications

Zarantoniello, M. *et al.* (2019). A six-months study on Black Soldier Fly (*Hermetia illucens*) based diets in zebrafish. *Scientific Reports* 9, 8598 (Chapter 2.1).

Zarantoniello, M. *et al.* (2020). Black Soldier Fly (*Hermetia illucens*) reared on roasted coffee by-product and *Schizochytrium* sp. as a sustainable terrestrial ingredient for aquafeeds production. *Aquaculture* 518, 734659 (Chapter 3.1).

Randazzo, B. *et al.* (2020). Can insect-based diets affect zebrafish (*Danio rerio*) reproduction? A multidisciplinary study. *Zebrafish* 17(5), 287-304 (Chapter 2.2).

Zarantoniello, M. *et al.* (2020). Zebrafish (*Danio rerio*) physiological and behavioural responses to insect-based diets: a multidisciplinary approach. *Scientific Reports* 10, 10648 (Chapter 3.2).

Zarantoniello, M. *et al.* (2021). Physiological responses of Siberian sturgeon (*Acipenser baerii*) juveniles fed on full-fat insect-based diet in an aquaponic system. *Scientific Reports* 11, 1057 (Chapter 4.1).

Randazzo, B. *et al.* (2021). *Hermetia illucens* and poultry by-product meals as alternatives to plant protein sources in gilthead seabream (*Sparus aurata*) diet: a multidisciplinary study on fish gut status. *Animals* 11(3), 677 (Chapter 4.2).

Zarantoniello, M. *et al.* (2021). Possible dietary effects of insect-based diets across zebrafish (*Danio rerio*) generations: a multidisciplinary study on the larval phase. *Animals* 11(3), 751 (Chapter 3.4).

Zarantoniello, M. *et al.* (2021). Application of laboratory methods for understanding fish responses to black soldier fly (*Hermetia illucens*) based diets. *Journal of Insects as Food and Feed* in press (Chapter 5.1).

Chemello, G. *et al.* (2022). Effects of Black Soldier Fly (*Hermetia illucens*) enriched with *Schizochytrium* sp. on zebrafish (*Danio rerio*) reproductive performances. *Aquaculture* 550, 737853 (Chapter 3.3).

Contribution to scientific conferences

Oral communications

Zarantoniello, M. *et al.* Physiological effects of insect-based diets during *Danio rerio* larval development. Aquaculture America 2020 - International Conference and Exposition, World Aquaculture Society 2020, February 9-12, Honolulu, Hawaii. (Chapter 3.1).

Zarantoniello, M. *et al.* Effects of new and sustainable aquafeed ingredients on zebrafish reproduction. 65° Convegno GEI-SIBSC Gruppo Embriologico Italiano, Società italiana di Biologia dello Sviluppo e della Cellula 2019, June 24-27, Ancona, Italy (Chapter 2.2).

Poster

Olivotto, I. *et al*. Effects of graded dietary *Hermetia illucens* inclusion levels on juvenile and adult zebrafish growth and welfare. Aquaculture America - International Conference and Exposition, World Aquaculture Society 2020, February 9-12, Honolulu, Hawaii (Chapter 3.2).

Awards

"SUSHIN Award" for the best innovative research project titled "Zero-waste production of alternative aquafeed ingredients for farmed fish culture" proposed within the SUSHIN project funded by Ager foundation (Coordinator Prof. Emilio Tibaldi, Department of Agri-Food, Environmental and Animal Sciences, University of Udine).

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