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The use of unconventional products in aquaculture, use of insects and macroalgae as feed additives for teleosts

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Content

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Abstract

One of the major challenges of the aquaculture sector is to replace most of fish meal and fish oil with more sustainable, cost-effective and environmental friendly ingredients. Insects and seaweeds are interesting candidates. Insects are extremely diverse nutritious organisms capable of massively growing on a variety of compounds, including organic wastes. Seaweeds are nutritious crops rich in bioactive compounds, which culture is associated with positive impacts to the environment. Early research on these unconventional ingredients is promising but in its infancy.

The major goal of the present doctoral thesis was to evaluate the effects of insect based diets and seaweeds on key fish biological responses and to improve the understanding of the mechanisms involved in fish nutrition and growth. A comprehensive knowledge at the histological, biochemical, spectroscopic, physiological and molecular level was achieved through a multidisciplinary approach. Taking advantage of fish models, that included zebrafish (*Danio rerio*), clownfish (*Amphiprion ocellaris*) and medaka (*Oryzias latipes*), information obtained in the present thesis may be used to generalize how several biological processes occur in related organisms.

Results from the first three experiments, on zebrafish and clownfish, revealed a good potential of insect based diets at even high inclusion levels, up to 100% in zebrafish larval rearing. Fish growth and survival were not affected by the new diets and no signs of intestinal inflammation were detected. In fact, clownfish fatty acid profile was profoundly affected by the novel ingredient, unlike zebrafish which showed molecular and biochemical capacity to compensate essential fatty acid deficiencies. Finally, results from the fourth chapter revealed that the seaweeds *Palmaria palmata* and *Saccharina latissima* did not induce any functional effect on the fish growth and gut health parameters of medaka.

Sommario

Una delle maggiori sfide del settore dell'acquacoltura è la sostituzione di farina ed olio di pesce con ingredienti più sostenibili, vantaggiosi dal punto di vista economico e sostenibili dal punto di vista ambientale. Gli insetti e le alghe si presentano come candidati interessanti da questo punto di vista. Gli insetti sono degli organismi estremamente eterogenei e capaci di crescere in maniera massiva su una varietà di composti, inclusi i rifiuti organici. Le alghe sono vegetali ricchi in composti bioattivi, la cui coltura è associata ad impatti positivi sull'ambiente. I primi studi su questi ingredienti non convenzionali hanno mostrato risultati promettenti, ma sono tuttavia ancora agli albori.

Lo scopo principale della presente tesi di dottorato è stato quello di valutare gli effetti di diete basate su insetti ed alghe sulle risposte chiave dei pesci per migliorare la comprensione dei meccanismi coinvolti nella nutrizione e nella crescita. Attraverso un approccio multidisciplinare è stato possibile ottenere una conoscenza di insieme a livello istologico, biochimico, spettroscopico, fisiologico e molecolare. Avvalendosi di pesci come modello sperimentale, come lo zebrafish (*Danio rerio*), il pesce pagliaccio (*Amphiprion ocellaris*) ed il medaka (*Oryzias latipes*), le informazioni ottenute nella presente tesi possono essere utilizzate per comprendere come diversi processi biologici avvengono in organismi simili.

I risultati ottenuti dai primi tre esperimenti, su zebrafish e pesce pagliaccio, hanno mostrato un buon potenziale di diete a base di insetti nell'allevamento larvale di zebrafish anche ad alte inclusioni e fino al 100%. La crescita e la sopravvivenza non è stata influenzata dalle nuove diete e non è stata osservata l'insorgenza di alcun segno di infiammazione a livello dell'intestino. La limitazione maggiore ha riguardato il contenuto in grassi degli insetti. Infatti, il profilo in acidi grassi dei pesci pagliaccio è stato profondamente influenzato da questi ingredienti innovativi, a differenza di quanto osservato in zebrafish il quale ha mostrato la capacità di compensare le mancanze in acidi grassi essenziali attraverso l'attuazione di meccanismi molecolari e fisiologici. Infine, i risultati riportati nel quarto capitolo, hanno messo in evidenza che le alghe Palmaria palmata e Saccharina latissima non hanno indotto nessun effetto funzionale sulla crescita e sui parametri di benessere intestinale in medaka.

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Introduction

Present issues related to aquaculture

The global population has dramatically increased since the beginning of the Industrial Revolution and now stands at 7.6 billion, projected to rise to 9.7 billion people by 2050 (United Nations, 2019). This rapid growth will bring central challenges regarding food production and food security. The animal production sector, but in particular aquaculture, has an increasing importance for meeting this challenge (FAO, 2018).

While aquaculture dates back millennia when culture of common carps (*Cyprinus carpio*) was developed in China (Nash, 2006), the industrialization of aquaculture did not occurred till recent years. Aquaculture is today the youngest and fastest-growing commercial food-production sector, with an almost exponential growth (FAO, 2003, 2018, 2019).

According to Tacon and Metian (Tacon and Metian, 2015), since 2000, aquaculture production exceeded capture fisheries production (Figure 1), and since then aquaculture production of animals and plants has grown at a mean rate of 5.8% (FAO, 2018, 2019), which is the highest among farmed animals (http://www.fao.org).

Conversely, capture fishery production has been relatively static since the late 1980s (FAO, 2018), despite increased worldwide fishing efforts (Bell et al., 2017). Fisheries reached a top production of 93 million tons in 1994 and since then has fluctuated between 89 and 97 million tons per year (FAO, 2018). This is probably consequence of overfishing, as showed by the FAO assessment of 600 marine fisheries (FAO, 2018).

Fish combined from capture fisheries and aquaculture is the largest source of animal protein in the world (Lucas et al., 2019). Seafood makes up 20%–25% of all animal proteins produced per year for human consumption (Lucas et al., 2019). With livestock production increasing at about 1.5% per year (FAO, 2003), aquaculture is increasing at 5.8% per year as the fastest-growing food production system globally (FAO, 2018).

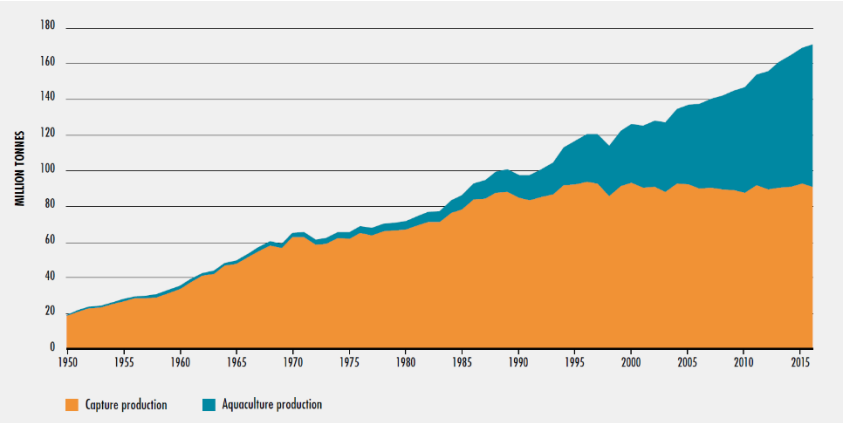

Figure 1. World capture fisheries and aquaculture (FAO, 2018).

Unfortunately, the dramatic growth of the aquaculture industry has been associated to a number of negative environmental and ecological impacts (Martinez-Porchas and Martinez-Cordova, 2012). Aquaculture activities are responsible of important large-scale conversion of coastal wetlands, mainly mangroves, coastal lakes and lagoons (Veettil et al., 2019), which provide habitat for a large number of fish, shellfish, and shrimp species at early life stages and thus serve as important breeding and nursery areas (Primavera et al., 2019). Changes in land use for aquaculture industry have resulted in degradation of important coastal lakes, reefs, mangroves, salt marshes, tidal creeks and lagoons that causes serious environmental damage (Rahman et al., 2013).

Effluents from aquaculture systems may have large amounts of uneaten feed and fish feces that may induce eutrophication (Rennie et al., 2019). They may also contain a variety of constituents that could cause negative impacts when released into the environment, especially chemicals (Chemello et al., 2016). In addition, disease issues have resulted from the high stocking density typical of aquaculture practices. Antibiotics overuse for therapeutic purposes, but also as growth promoters (they may promote growth and improve feed efficiency), have led to serious antibiotic resistance. In Europe the widespread use of antibiotics has been limited to therapeutic use by veterinary prescription since 2006. There is an urgent worldwide need to develop novel antimicrobial agents (Wang et al., 2016).

Feed production in aquaculture. Fish meal and fish oil as main ingredients

Aquaculture production is dependent upon the provision and supply of feed or nutrient inputs to the culture system. Feed costs account for over 50% in most aquaculture

operations (Lucas et al., 2019), with protein as the principal ingredient and the most expensive component. Protein levels in aquafeeds generally range from 30% to 35% for shrimp (Lee and Lee, 2018), 36% to 44.12% for catfish (Jayant et al., 2018), 15% to 56% for tilapia (Liu et al., 2017) and 40% to 45% for trout and other marine finfish (Craig, 2017). In general, protein requirements are typically lower for herbivorous fish and omnivorous fish than they are for carnivorous fish (Craig, 2017).

Fish meal is considered an ideal protein source for aquafeeds, being easily digestible and supplying almost a perfect balance of the essential nutrients required to ensure good health and low feed conversion ratios of farmed fish (Tacon and Metian, 2013). It has been traditionally considered a cost-effective protein source in feed formulation (Tacon and Metian, 2013). Fish meal is typically produced from fish species not used for direct human consumption, or from the byproducts of seafood processing (Cashion et al., 2017).

The fish and crustacean aquaculture sector, estimated at over 50.6 million tons or 55.9% of total global aquaculture production in 2012 (Tacon and Metian, 2015), has been the largest consumer of captured non-food products for over a decade (Naylor et al., 2009), either in the form of fish meal and fish oil used within industrially compounded aquafeeds or in the form of whole/processed fish used as a direct feed or within farm-made aquafeeds (Hasan, 2012). Fish meal and fish oil prices are expected to vary from US $1100 to $1 500 per ton during 2019 (https://thefishsite.com/).

Lipids are required by fish as a source of available energy, as structural components of biomembranes, carriers of fat-soluble vitamins, precursors of eicosanoids, hormones and vitamin D, and as enzyme co-factors (Izquierdo and Koven, 2011). Marine fish oils (FO) have traditionally been used as the sole dietary lipid source in commercial fish feeds given their high availability, competitive price and good essential fatty acids content (Turchini et al., 2009).

Fish meal and fish oil are mainly obtained from wild-caught small marine fish species such as sardine, anchovy, herring, capelin, mackerel, etc., with Chile and Peru as the world's largest suppliers (Turchini et al., 2009). Although annual global production has been relatively constant over decades, during El Niño events, production in Peru and Chile is substantially reduced (FAO, 2018). These countries account for about one-third of global production, and unfortunately their production is some years uncertain by the effects that El Niño events exert on this part of the Pacific Ocean (FAO, 2018). Thus

changes in their production of fish meal and fish oil greatly affect global supplies and prices (Hardy and Tacon, 2002; Shepherd and Bachis, 2014).

Problems related to fish meal and fish oil and the search for other commodities

Given the current demand of fishmeal and fish oil by aquaculture, one of the major challenges faced by aquaculture is that current supplies are not sufficient to support the required expansion of aquaculture production (Lucas et al., 2019). With the wild marine fish stocks at or above maximum sustainable yield, aquaculture can no longer rely on oceanic resources for the manufacturing of aquafeeds, and such feed options are no more sustainable from both, economic and ecologic point of view (Naylor et al., 2009). The price of fish meal and fish oil are expected to continue increasing in the near future. Nevertheless, in addition to the growing world population, according to the growing global fish consumption (FAO, 2018), the demand for aquafeed is expected to increase even more due to growing health awareness and population growth. The ethics of using fishery resources with food-grade potential (pelagic fish, such as mackerel, hake, whiting, pilchards, sardines and capelin) for animal feeding rather than for direct human consumption is a controversial issue (Tacon and Metian, 2009).

Other commodities: Positive and negative aspects of each

In order to expand and maintain the aquaculture industry competitive, intensive research has been globally conducted to develop less expensive alternative sources of lipids and proteins (Bandara, 2018). Research to develop substitutes for these feed ingredients has focused on commodities ranging from plant products (especially soybeans) to meat byproducts (such as blood meal and bone meal), including microbial proteins (Bandara, 2018).

Due to higher abundance and lower price compared to fish meal, ingredients of plant origin have received the most attention and, in fact, many of them are nowadays widely used in aquafeed formulation (Ayadi et al., 2012). These include grains (e.g. wheat and corn), oilseeds (e.g. soybean, sunflower, rapeseeds, cottonseed), and pulses (e.g. beans, lupins, and peas) (Bandara, 2018). The nutritional value of these feedstuffs, however, varies considerably according to numerous factors, including the feedstuff itself, the variety, season, and processing method, which impose several problems for their use (Ayadi et al., 2012). By far the most important plant ingredient in aquaculture and animal farming is the soybean meal due to its high protein content (crude protein ranging from

44% to 48%), high digestibility and the best amino acid profile among vegetable protein sources available (Herman and Schmidt, 2016). Concentrations of the 10 essential amino acids and tyrosine, are generally lower in soy bean meal than in fish meal with the exception of cystine, which is present at higher concentrations in soy bean meal. Carbohydrates in soybeans include complex forms, such as raffinose and stachyose which are not digestible by the fish. Canola (rapeseed) meal is mainly an oil-source (40–45% dry matter, DM), but the rapeseed meal obtained after extraction of oil is a good protein-source with protein content varying between 32% and 45% DM (Burel et al., 2000). It contains a well-balanced amino acid profile compared to soy bean meal, but with a high fiber content (Gatlin et al., 2007). Conversely, peas and lupin have a low protein content (<25% DM) and a high starch content (>50% DM) (Burel et al., 2000; Gatlin et al., 2007). The essential amino acids content is comparable to fish meal but lysine and methionine are limited. Similarly, whole grain wheat contains a low protein content (12.9%) and a high starch composition (typically >70%). Lysine and methionine are relatively minor constituents of the total, 3% and 1.5% respectively.

Unfortunately, plant proteins present inherent disadvantages as a result of their defense against herbivores (Ayadi et al., 2012). Most of the potential, alternative, plant-derived nutrient sources are known to contain a wide variety of antinutritional substances (Krogdahl and Bakke, 2015). These include protease inhibitors, phytates, glucosinolates, saponins, tannins, lectins, oligosaccharides and non-starch polysaccharides, phytoestrogens, alkaloids, antigenic compounds, gossypols, cyanogens, mimosine, cyclopropenoid fatty acids, canavanine, antivitamins, and phorbol esters (Krogdahl and Bakke, 2015). In addition, they also contain high fiber levels, inadequate fatty acids and amino acid profiles (typically low in methionine)(Gai et al., 2012), and low palatability (Gatlin et al., 2007). Not surprisingly adverse effects are frequently reported on fish feed intake, nutrient-energy digestibility, retention efficiency and thus low growth performance and survival (Burel et al., 2000; Johri et al., 2011; Booman et al., 2018).

Some limitations, but not all, of plant ingredients can be overcome through the use of technology. For example, some antinutritional factors can be inactivated or reduced by heat treatment, extrusion and fractionating of the crops, while heat stable antinutrients can sometimes be removed by enzymatic treatment, although often at the expense of negative impacts in protein, carbohydrate and lipid digestion by the fish (Krogdahl and Bakke, 2015). As regards their nutritional imbalances, the amino acid profile may be

improved by supplementation of essential amino acids, such as lysine and methionine (Jiang et al., 2016). The combined use of one or more plant ingredients can be also applied to correct imbalances of the amino acid plant profile, such as a mixture of corn gluten (high in methionine but low in arginine and lysine) and soybean (high in arginine and lysine, low in methionine) meal (Lall and Anderson., 2005). However, these kind of combinations could be challenged by the interaction of different antinutritional factors in plant ingredients (Prabu et al., 2017).

Since the last decade, plant oils are widely used in aquaculture due to their lower cost and higher availability compared to fish oil (Turchini et al., 2009). Soybean, palm, rapeseed and sunflower oil are the major sources (Ayisi et al., 2019). Vegetable oils are rich sources of n-6 and n-9 fatty acids (FA), mainly linoleic acid (18: 2n-6) and oleic acid (18:1 n-9), with the exception of linseed oil, which is rich in α-Linolenic acid (18:3 n-3) (Turchini et al., 2011). Unfortunately most vegetable oils are relatively poor sources of n-3 FAs in comparison to marine fish oil, and this remains as the main limitation for aquaculture use (Ayisi et al., 2019). In general, research has reported that vegetable oils can replace substantial amounts of fish oil in the diets of many fish species without affecting growth or feed efficiency, provided that adequate amounts of specific essential fatty acids (EFA) are supplied in the diet (Gatlin et al., 2007).

Terrestrial animal byproducts possess advantages compared with vegetable sources (Ayadi et al., 2012). Animal sources generally contain highly digestible protein with good amino acid profiles and are naturally free from antinutritional compounds (Naylor et al., 2009). Animal byproducts include meat and bone meal from swine and cattle as well as blood meal, poultry byproduct meal and feather meal (Ayadi et al., 2012). They have been extensively used in fish feeds for several decades in Europe prior to be banned in 2001 as a consequence of the bovine spongiform encephalopathy and the transmissible spongiform encephalopathies issues (EC No 999/2001) (Naylor et al., 2009). Then in 2013, it was re-admitted the use processed animal protein from non-ruminant animals (poultry and pigs), offering new opportunities for a more sustainable and conscious application.

Another interesting kind of fish meal alternative is the use of micro-and-macro algae. Microalgae represent a natural source of pigments, antioxidants and bioactive compounds which give them functional properties in addition to their basic nutritional value (Spolaore et al., 2006). Due to a minimal overall environmental impact compared to most

conventional feed commodities (Muller-Feuga 2004; Tibaldi et al., 2015), dried microalgae biomass has been proposed as potential raw materials in partial substitution for fish-derivatives in fish feeds.

The need to identify alternative sustainable ingredients and the European Commission recommendations.

Despite huge efforts to find new alternative ingredients for the aquaculture industry, there is still urgency to find better alternatives given that vegetable feedstuffs have the unfavourable characteristics previously described, while animal protein, which has high digestibility and a good balance between essential and nonessential amino acids, possess several restriction on their use. Aquaculture is still highly depended on the use of fish meal and current supplies are not sufficient to support the required expansion of aquaculture production (Naylor et al., 2009).

Insects as a valuable new aquafeed ingredient. Advantages, disadvantages.

In the intensive search for new aquafeed ingredients, researches have turned their attention to insect meals as a sustainable animal source for feed production (FAO, 2013; Lock et al., 2018). Insects are the most diverse living organisms. With an estimated total number of species of around 5 to 10 million (Ødegaard, 2000), at least 1 681 insects can potentially be used as food and feed (Ramos-Elorduy, 2008). Insects may be found in nearly all terrestrial environments where they may grow at high rates on a great variety of organic materials, including organic wastes and could thus be industrially grown on organic side streams, reducing environmental contamination and transforming waste into high value biomass that can replace increasingly expensive feed ingredients, such as fish meal and fish oil.

Insect meal is very interesting from a nutritional point of view, with a high protein content and good amino acid balance. The average protein content of insects varies between 40 and 60%, similar to soy meal levels (50% crude protein), but still lower than fish meal levels (73.0% crude protein). The order Coleoptera has shown the higher crude protein levels, between 60 and 70 %, while Diptera has shown values ranging from 40 to 50% crude protein. Factors such as developmental stage and rearing substrate of insects can affect these values. For example, larvae of yellow mealworm (*Tenebrio molitor*) grown on wheat, grain and carrots showed a protein content of 83.0 g kg^{-1} protein, but of 598.1 g kg^{-1} when grown on a diet enriched with fatty acids, vitamins E, β-carotene

(Nogales-Mérida et al., 2018) This opens new opportunities for modulating their nutrient content.

In addition, their amino acid profile is also promising. From the ten essential amino acids important for fish (NRC, 2011), most are provided by insect proteins. This is not surprising given that they are often part of the natural fish diet (Henry et al., 2015). However, relative to fish meal, insect meal is deficient in the amino acids histidine, lysine and threonine, but better in lysine methionine and tyrosine than soy meal (Barroso et al., 2014).

Dipterans have been recognized as the best candidates as concerns the amino acid profile, with a histidine, lysine and threonine proportion similar to fish meal (Barroso et al., 2014). However, Diptera exhibits a relative deficit in leucine that does not occur in Orthoptera or Coleoptera. Enrichment with limiting amino acids or a combination of complementary insect preparations may be a good approach to supply a desirable balanced amino acid profile. The digestibility of such amino acids is generally better than plant proteins and may in addition be subjected to manipulation, particularly by processing methods such as drying and grinding (Nunes et al., 2014).

Insects are also a great source of lipids. As energy reserves, especially during the immature stages (larva, pupa, nymph), insects bioaccumulate lipids that support the development during certain non-feeding periods of their life cycle (e.g. diapause, metamorphosis, etc.). Compared to the protein content, the lipid content is highly variable (Barroso et al., 2014). Whereas the larvae of the boas rhinoceros beetle (*Oryctes boas*) showed a lipid content % (dry matter) of 1.5 (Okoli et al., 2019), the edible larvae of a Mexican beetle showed a lipid content % (DM) of 56.1 (Ramos-Elorduy, 2008). The larvae of coleopterans generally have a high amount of fat, often exceeding 25% (Barroso et al., 2019). The black soldier fly (BSF) shows a great lipid bioaccumulation capabilities, which range from 7 to 39%, depending on the feeding substrate of the larvae (Tschirner and Simon, 2015; Barragan-Fonseca et al., 2017). Unfortunately, the fatty acid composition of insects are known to be poor in polyunsaturated fatty acids (PUFAs) which are essential in fish nutrition (Barroso et al., 2017). Saturated fatty acids (SFA) and particularly lauric acid (12:0) made up most lipid fraction (Vargas et al., 2018; Zarantoniello et al., 2018; Belghit et al., 2019). In particular, the FA composition of BSF larvae is characterised by a high lauric acid percentage, which reaches approximately half of the total FA, as well as palmitic (16:0), myristic (14:0), oleic (18:1n9), linoleic

(18:2n6), and α-linolenic (18:3n3) acids, with α-linolenic usually in percentages less than 10% of the total FA (Vargas et al., 2018). It has been demonstrated that the lipid content and the fatty acid composition largely depend on the insect diet. Currently, most research is focusing on testing different insect diets to enhance the insect nutritional value and thus better fit fish requirements.

Insects are also a good source of vitamins and minerals. The majority of insects show high amounts of potassium, calcium, iron, magnesium (Schabel, 2010), and selenium (Finke, 2002). Bee brood is rich in vitamin A and D, whereas caterpillars are especially rich in Vit B1, B2 and B6 (Schabel, 2010). Interestingly, minerals and vitamins can also be enriched through the insect diet (Liland et al., 2017).

One of the possible negative aspect of insect meal as aquafeed ingredient could be due to their high content of the polysaccharide chitin found in the cuticle of insects, which might affect the digestibility and the utilization of nutrients (Kroeckel et al., 2012; Marono et al., 2015). However, the effects of the chitin in fish feeds is still not fully understood, and results are controversial. Despite the presence of chitinolytic activity in fish by specific enzymes (Fines and Holt, 2010; Kawashima et al., 2016; Zarantoniello et al., 2019), or by exogenous microbiota activity (Zhou et al., 2013; R Magalhães et al., 2017; Gasco et al., 2018), they have limited digestive capacity for chitin and it has been demonstrated that diets rich in chitin can have a negative impact on food intake, availability, digestibility, and absorption of nutrients and thus growth performances (Gopalakannan and Arul, 2006; Harikrishnan et al., 2012; Kroeckel et al., 2012; Dumas et al., 2018; Rapatsa and Moyo, 2019). Access of chitinases or proteinases to their substrates may be limited by the complex cuticle of insects which is composed of chitin in a matrix of proteins, lipids and other compounds (Kramer et al., 1995). In addition, high chitin inclusions in aquafeeds may induce intestinal inflammation and a reduction in fish welfare and growth (Kroeckel et al., 2012; Li et al., 2017; Zarantoniello et al., 2018).

However, chitin may also exert a positive effect on the functioning of the immune system. Chitin, which is present also in pathogens such as fungi, bacteria and parasites, may activate pattern recognition receptors in the fish innate immune system, stimulating the production of various cytokines and immune mediators (Da Silva et al., 2008). Recent findings showed that chitin, or its chitosan constituents, when used in moderate inclusions enhanced lysozyme antibacterial activity in European sea bass (Henry et al., 2018), and

improved survival rates of the pacific white shrimp (*Litopenaeus vannamei*) after challenged with pathogenic bacteria (*Vibrio parahaemolyticus*) (Motte et al., 2019).

Nevertheless, several methods can be employed to get rid of chitin (Henry et al., 2015); however, a better understanding of the mechanisms involved in chitin digestion and its interaction with other nutrients, as well as the fish responses, will open new opportunities in fish nutrition, with the high energy content (17.1 kJ g^{-1}) and high abundance of chitin (Fines and Holt, 2010).

In addition to the nutritive value of insects, numerus bioactive compounds in insects can be used to boost animal health (Gasco et al., 2018). More than 150 peptides with antimicrobial and antifungal properties have been isolated from insects (Vogel et al., 2018). These peptides, along with chitin and short-chain fatty acids such as lauric acids (abundant in insect) (Dayrit, 2015; Li et al., 2016; Belghit et al., 2019), not only exhibit antimicrobial activity, but they can also stimulate the immune response (Harikrishnan et al., 2012; Zhang et al., 2012; Su et al., 2017; Henry et al., 2018; Xiao et al., 2018) in fish and help shaping their microbiota, resulting in improved gut health, nutrient digestibility and growth performance. This is of great relevance with the rising antibiotic resistance concerns (Povolo and Ackermann, 2019).

State of the art - insect meal in aquaculture

The use of insect meal in aquafeeds is presently a hot topic. A growing number of insect species are being studied as fish meal replacements (Table 1). By far the most studied insect species are the black soldier fly (*Hermetia illucens*) and the yellow mealworm (*Tenebrio molitor)*, which are two of the seven insect species that the European Commission recently approved for their use in aquafeeds (Regulation 2017/893/EC), following the European Food Safety Authority (EFSA) scientific opinion on the use of insects as food and feed (EFSA, 2015). According to Henry et al. (2015) at least 24 different fish species have been tested in nutritional experiments using insects, with Atlantic salmon and rainbow trout as the most studied ones. Aside from fish, insect meals are also interesting for shrimp production (*Litopenaeus vannamei*) (Panini et al., 2017b, 2017a; Motte et al., 2019). Nutritional studies in fish have included herbivorous and omnivorous fish such as catfish, tilapia and carp, and carnivorous species such as Atlantic salmon and rainbow trout. While most studies reported insect meal inclusion levels below

50%, several authors have reported complete replacements with promising results (Bondari and Sheppard, 1987; Ng et al., 2001; Lock et al., 2016; Vargas et al., 2018).

Table 1. Insect species tested in aquafeeds.

Order	Common name	Latin name	Reference
Orthoptera	Variegated grasshopper	*Zonocerus variegatus*	(Alegbeleye et al., 2012)
Orthoptera	Painted grasshopper	*Poekilocerus pictus*	(Johri et al., 2011)
Orthoptera	Migratory locust	*Locusta migratoria*	(Emehinaiye, 2012)
Orthoptera	Indian house cricket	*Gryllodes sigillatus*	(Józefiak et al., 2019)
Orthoptera	Jamaican field cricket	*Gryllus assimilis*	(Fontes et al., 2019)
Orthoptera	House cricket	*Acheta domesticus*	(Irungu et al., 2018)
Blatodea	Turkestan cockroach	*Blatta lateralis*	(Józefiak et al., 2019)
Blatodea	Cinerea cockroach	*Nauphoeta cinerea*	(Fontes et al., 2019)
Blatodea	Hissing cockroach	*G. portentosa*	(Fontes et al., 2019)
Blatodea	Termites	*Macrotermes longifilis*	(Sogbesan and Ugwumba, 2008)
Coleoptera	Yellow mealworm	*Tenebrio molitor*	(Motte et al., 2019)
Coleoptera	Asiatic rhinoceros beetle	*Oryctes rhinoceros*	(Okoli et al., 2019)
Coleoptera	Superworm	*Zophobas morio*	(Fontes et al., 2019)
Lepidoptera	Domesticated silkworm	*Bombyx mori*	(Chen et al., 2016)
Diptera	Chironomid flies	-	(Roncarati et al., 2019)
Diptera	Common house mosquito	*Culex pipiens*	(Ostaszewska et al., 2011)
Diptera	Black soldier fly	*Hermetia illucens*	(Vargas-Abúndez et al., 2019)
Diptera	Common housefly	*Musca domestica*	(Xiao et al., 2018)

In Atlantic salmon (*salmo salar*), replacing fish meal up to 100% with insect meal have been achieved by most studies, without any negative effect on fish growth performance, feed intake, feed utilization, nutrient digestibility, intestinal trypsin activity, liver and gut health and inflammatory transcripts (Lock et al., 2016; Belghit et al., 2018, 2019; Y Li et al., 2019). However, a possible damage due oxidative stress, as revealed by a transcriptomic study on the cellular stress response in salmon head kidney was reported (Stenberg et al., 2019), as well as a possible negative impact on nutrient digestibility due to a reduction in a brush border enzyme responsible of breaking down peptides into amino acids was observed (Belghit et al., 2018). Similarly, in rainbow trout very high insect inclusion levels have been achieved without negative impacts on the fish performance, although most of these studies were performed using generally lower insect inclusion

levels compared to salmon. Studies replacing fish meal up to 50% with insect meal reported good results in terms of survival, growth performance, condition factor, somatic indexes and feed utilization (Sealey et al., 2011; Renna et al., 2017; Stadtlander et al., 2017; Dumas et al., 2018). However with similar inclusion levels, other studies have highlighted negative impacts on fish growth performance, feed efficiency and apparent digestibility of proteins (St-Hilaire et al., 2007; Renna et al., 2017; Dumas et al., 2018). Good results have also been observed for Jian carp (*Cyprinus carpio* var. Jian) (Zhou et al., 2018), European seabass (*Dicentrarchus labrax*) (Rui Magalhães et al., 2017), Clownfish (*Amphiprion ocellaris*) (Vargas-Abúndez et al., 2019) and Pacific white shrimp (*Litopenaeus vannamei*) (Panini et al., 2017a), whereas impaired lipid metabolism, growth and feed efficiency have been reported for zebrafish larvae (Zarantoniello et al., 2018), European seabass juveniles (Gasco et al., 2016) and juvenile pearl gentian grouper (*Epinephelus lanceolatus* × *Epinephelus fuscoguttatus*)(Song et al., 2018). From all these studies, it is clear that the central issues of using insect meal is their poor PUFAs content, which affects the fish nutritional quality, as well as the presence of chitin which possibly cause of the observed reduced food intake, digestibility, and nutrient absorption in cultured fish.

As previously described, chitin is a primary component of the insect exoskeletons and is presently considered as an insoluble fiber, similarly to vegetable fibers, since most fish cannot digest it and can thus reduce food intake and nutrient absorption. However, several studies have shown that chitin or its chitosan constituents, when used in moderate inclusions, resulted in increased fish immune response (Esteban et al., 2000; Gopalakannan and Arul, 2006; Harikrishnan et al., 2012; Ringø et al., 2012; Zhang et al., 2012; Henry et al., 2018) and in a positive microbiota modulation (Askarian et al., 2012; Zhou et al., 2013; Bruni et al., 2018; Stenberg et al., 2019). The effects of the chitin in fish feeds is thus still controversial and not fully understood, and more research is needed.

Chitin effects at the histological level are poorly studied. While first investigations have detected no morphological changes between fish fed with insect diets and control diets (Lock et al., 2016; Rapatsa and Moyo, 2017; Renna et al., 2017; Vargas et al., 2018), other researchers using high inclusion levels of HI meal in rainbow trout (*Oncorhynchus mykiss*), carp (*Cyprinus carpio*) and clownfish (*Amphiprion ocellaris*) diets detected intestine morphology changes, which is indicative of impaired nutrient absorption (Li et al., 2017; Dumas et al., 2018; Vargas-Abúndez et al., 2019).

Several are the possible explanations of the above mentioned discrepancies. Firstly, different fish species have different chitinolytic activity and this may yield to different chitin digestion capabilities. Some species, like turbot (*Scophthalmus maximus*), do not have any detectable chitinolytic activity (Kroeckel et al., 2012), while others, like cobia (*Rachycentron canadum*), have high chitinolytic activities capable of digesting chitin (Fines and Holt, 2010). Secondly, even if it has been demonstrated that fish may upregulate chitinases genes with increasing dietary insect meal (Vargas et al., 2018; Zarantoniello et al., 2018, 2019), and that fish like African sharptooth catfish (*Clarias gariepinus*) (Rapatsa and Moyo, 2019), increase chitinolytic activity with increasing dietary insect meal, that does not necessarily means that chitin is digested. Thirdly, the percentage composition of chitin varies among different insect species (Caligiani et al., 2018; Y.-S. Song et al., 2018), and while at low dietary levels they may act as immunomodulators (Gopalakannan and Arul, 2006; Henry et al., 2018) and as probiotics (Bruni et al., 2018; Antonopoulou et al., 2019; Huyben et al., 2019; Terova et al., 2019), at high dietary inclusion levels they may act as irritants and antinutritional factors (Li et al., 2017; Dumas et al., 2018; Vargas-Abúndez et al., 2019). Finally, the absence of intestine inflammation may be related also to the insect fatty acid composition. Many insect species are known to be rich in SFA (especially lauric acid), that have been reported to improve gut health because of their intestinal anti-inflammatory, antibacterial (Feng et al., 2018; Henry et al., 2018; Vargas et al., 2018) and antiviral activity (Ponnuvel et al., 2003; Dayrit, 2015; Liland et al., 2017). In this regard, a recent study by Li et al. (2019) found that the insect diet tended to improve gut function in pre-smolt Atlantic salmon by reducing excessive lipid deposition within the enterocytes of the pyloric caeca.

As already pointed out, a second limiting factor of insects as new ingredient in aquafeeds is their FA profile that does not always match the nutritional requirements of fish. On this regard, increasing evidence showed that increasing levels of insect meal inclusion caused a dramatic change of the FA profile in fish, with a decrease in eicosapentaenoic acid (EPA) and docosahexaenoic acid (DHA) and a consequent decrease in the n3/n6 fatty acid ratio (Belforti et al., 2015; Barroso et al., 2019; Vargas-Abúndez et al., 2019). This fatty acid unbalance has been reported to induce liver damage in different fish species. In zebrafish larvae, a fish meal replacement by BSF larvae meal of 50%, induced a severe lipid steatosis in the liver, even if the tested diets were isolipidic (Zarantoniello et al., 2018). Previous observations on the importance of PUFAs deficiency and high n-6/n-3

ratios on the development of hepatic steatosis, led the authors hypothesize that the observed lipid steatosis could be related to the quality (n-6/n-3 ratio) rather than the quantity of the dietary fat provided through the three different diets. A similar observation was pointed out by Vargas et al. (2018), in which BSF larvae grown on coffee byproducts, displayed a FA profile rich in SFA poor in PUFAs, that induced zebrafish larval fatty liver. In line with this, high inclusion levels of mopane worm meal induced liver morphological damage in the african sharptooth catfish (*Clarias gariepinus*) that was possibly associated to decreased enzyme activity (Rapatsa and Moyo, 2019). In other studies, a dietary inclusion of BSF larvae, cricket and maggot meal resulted in higher activity and gene expression of the antioxidant system and an increased expression of hepatic heat-shock protein of carp and African catfish (superoxide dismutase and catalase in serum and liver) (Taufek et al., 2016; Li et al., 2017). Similarly in rainbow trout an unbalance of oxidative homeostasis in liver and kidney associated to HI dietary inclusion (50% fish meal replacement) was reported (Elia et al., 2018).

Some of these stress responses were not always accompanied by pathological conditions at the histological level. It is possible that the undesirable SFA, medium-chain FA (in particular of lauric acid), play a beneficial nutritional and therapeutic role. At this regard, a recent study by Belghit et al. (2019) in freshwater Atlantic salmon, as previously suggested by other studies, confirmed a decreased liver triacylglycerol content, likely due to the rapid oxidation and low deposition of the medium-chain fatty acid lauric acid, associated to good feed intake and digestibility values. In addition, the fact that the whole body fatty acid composition of salmon generally reflected that of the diets without negatively impact fish health, as observed in other species such as the blackspot sea bream (*Pagellus bogaraveo*) (Iaconisi et al., 2017) and zebrafish (Zarantoniello et al., 2019), suggest that dietary insect meal might have a protective effects in the liver of Atlantic salmon.

However, producing fish in line with welfare standards but with a low nutritive value (low in n-3 PUFAs), as evidenced by numerous studies on the fish lipid quality after fed insect diets, is detrimental for human nutrition. Efforts to increase the insect unsaturated FA content manipulating the rearing substrate have so far tested mixture of vegetable and fruit (Meneguz et al., 2018; Vargas et al., 2018); chicken feed, vegetable waste, biogas digestate, and restaurant waste (Spranghers et al., 2017); wheat middlings, dried distillers grains with soluble (as a protein rich substrate) and dried sugar beet pulp (as a fiber rich

substrate) (Tschirner and Simon, 2015), fish offal (St-Hilaire et al., 2007; Barroso et al., 2019); seaweeds (Liland et al., 2017; Swinscoe et al., 2019) and byproducts obtained from roasting coffee process (Vargas et al., 2018). All these with various degrees of success. Clearly, BSF larvae have the capacity to accumulate PUFAs from the feeding substrate, but at various efficiency rates. For instance, while BSF larvae fed only fish offal during 12 days accumulated 12.1% EPA + DHA (Barroso et al., 2019), larvae fed fish offal + cow manure (50% fish offal and 50% cow manure) during 21 days accumulated 2.25% EPA + DHA (St-Hilaire et al., 2007), and larvae fed seaweed that contained 6 % EPA + DHA, accumulated only 16 % of the FAs provided through the seaweed (Liland et al., 2017). As suggested by Barroso et al. (2019), BSF larvae may show different bioaccumulation capabilities depending on the differential metabolic homeostasis in the insect tissue. Indeed, not only the biochemical composition of the insect substrate affect the insect biochemical composition but also other factors such as physical properties of the substrate such as fiber content and particle size (Barragan-Fonseca et al., 2017; Liland et al., 2017; Spranghers et al., 2017).

Macroalgae as novel feed additive and fish meal replacement

Seaweeds (marine macroalgae) represent another innovative protein source for aquaculture sustainability (Rajauria, 2015). Seaweeds are primary producers that grow by absorbing dissolved nutrients, thus removing excess nutrients from the water (Neori et al., 2004). They play key ecological roles in coastal ecosystems by supporting food webs, and providing habitats for entire ecosystems. Seaweed aquaculture requires no fresh water, chemical fertilizer or arable land, and can be integrated in multi-trophic aquaculture systems, playing a key role for circularity in the use of natural resources (Zhang et al., 2019). With more than 6000 species described and remarkable biological diversity they represent an underutilized feed source in aquaculture (Lindsey White and Wilson, 2015).

Seaweeds naturally contain high levels of protein, ranging from 5% to 35% of its dry weight with relatively well-balanced amino acid profiles (Peng et al., 2015). The red seaweed *Palmaria palmata* is commonly found in Europe and America and is rich in protein content (9%–25%, depending on the time of harvest). Lipids represent about 1–5% of algal DM. Despite the low level of total lipids, much of this lipid content is made up of PUFAs, which are essential for the growth of many marine animals (Rajapakse and Kim, 2011). Seaweeds are rich in complex carbohydrates, many of which are undigestible

by the fish and represent thus potential prebiotics (Hindu et al., 2019). They are also an excellent source of vitamins such as vitamins A, B, C, D, and E, riboflavin, niacin, pantothenic acid, and folic acid as well as minerals such as iodine, calcium, phosphorus, sodium, and potassium. They have more than 54 trace elements, required for physiological functions, in quantities greatly exceeding land plants (Circuncisão et al., 2018). The composition of seaweeds is known to vary according to the season, the type of species, and the sampling techniques used (Circuncisão et al., 2018).

One of the most interesting features of seaweeds is the innumerable secondary metabolites they produce, which confer them functional properties such as antibacterial, antioxidant, antiinflammatory and antiviral properties (Angell et al., 2016). These metabolites include monoterpenes, sesquiterpenes, diterpenes, meroterpenoids, C15-acetogenins, phlorotannins, and steroids (Kadam et al., 2015).

Widely studied seaweed species for aquafeed formulation include *Ulva* sp., *Undaria* sp., *Ascophyllum* sp., *Porphyra* sp., *Sargassum* sp., *Polycavernosa* sp., *Gracilaria* sp., and *Laminaria* sp. Generally, inclusion levels in compound feeds for fish are low, since over 10% inclusion levels negative effects on fish growth performance have been observed (Sotoudeh and Jafari, 2017; Shi et al., 2019; Wang et al., 2019). Such growth reduction may be due to reduced feed intake (low palatability) and the relative low nutritive value of some seaweeds. Conversely, seaweeds can provide complete or partial protein replacements in species that naturally include large proportions of seaweed in their diets, such as abalone (Kemp et al., 2015), sea urchins (Shpigel et al., 2018), but also in prawn (Felix and Brindo, 2014). As regards fish nutrition, most studies have explored the potential of seaweeds as functional ingredients, testing inclusion levels around 5%.

As regards palatability, seaweed contains dimethyl-beta-propionthein, dimethyl sulfonyl propionate (DMSP), and some amino acids that may act as an attractant for fish and shrimp (Cruz-Suárez et al., 2009; Sotoudeh and Jafari, 2017). In a trial in juvenile rainbow trout fed the red seaweed *Gracilaria pygmaea* at dietary supplementation of 6% for 7 weeks, increased feed intake and decrease feed conversion ratio, compared to higher inclusion levels (12%) or a reference diet without the seaweed was reported (Sotoudeh and Jafari, 2017). In another recent study aiming to test dietary *Sargassum horneri*, a brown macroalgae, on juvenile black sea bream *Acanthopagrus schlegelii*, an optimal inclusion level of 6% promoted an increasing of total protein and globulin, decreasing of total cholesterol and triglyceride, and a better hepatic antioxidant status and immunity of

fish. Similar results were reported by Zhu et al. (2017) in white spotted snapper *Lutjanus stellatus*, which were fed diets supplemented with 5% *S. horneri*. Such effects were attributed to the presence of the immune stimulator fucoidan, an abundant polysaccharide in the cell wall of brown macroalgae, which has been reported to contain antioxidant, antibacterial, antiviral, antitumor, and immune enhancement properties (Telles et al., 2018). However, as it is the case for many other bioactive compounds such as carrageenans (abundant in red macroalgae) and ulvans (abundant in green macroalgae), research is needed to elucidate whether the bioactive compounds in seaweeds are responsible for their attributed benefits and as well as their modes of action.

Seaweeds as any other novel ingredient, present challenges and limitations. One of the main limiting factors of using seaweeds as a protein replacement is their low concentration of essential amino acids on a whole basis which is not adequate as a protein component of compound diets for fish (Angell et al., 2016). Their successful use requires of processing methods to concentrate protein. The presence of antinutrional factors, such as saponins, tannins, phytic acid, xylans, agar and alginates may negatively affect the nutritional quality and nutrients digestion/absorption of seaweeds and consequently, may reduce growth performance (Rajauria, 2015).

Model organisms

The present Ph.D. project was conducted using strategically three key fish models, a freshwater, and euryhaline and a marine one, with the aim of understanding the effects of unconventional products on the fish performance, physiological response, gut health as well as welfare of fish.

Danio rerio

Class: Actinopterygii

Order: Cypriniformes

Family: Cyprinidae

Subfamily: Danioninae

Genus: Danio

Zebrafish (*Danio rerio*) is a small tropical freshwater fish indigenous to South Asia. It is an emerging vertebrate model in the fields of biomedical, developmental biology, genetics, toxicology, and aquaculture (Chemello et al. 2016).

Zebrafish is an attractive model due to its small size, high fecundity, short generation time, rapid development (hatching occurs after 2-3 days post fertilization), external fertilization, translucent embryos (suitable for observation of internal organs with conventional microscopy) and an extensive amount of information made available by genome and transcriptome sequencing (Teraoka et al., 2003). With all these advantageous biological traits combined with recently established protocols and molecular tools, zebrafish represents a unique platform for uncovering the genetic and physiological regulatory networks underlying the organism response to innovative diets. Starting out as a model for developmental biology, today zebrafish may be a valid model used to generalize how several biological processes occur in conventional aquaculture species like trout, turbot, salmon, gilthead seabream and European seabass, contributing toward improving our understanding of the mechanisms involved in nutrition and growth.

Amphiprion ocellaris

Class: Actinopterygii

Order: Persiformes

Family: Pomacentridae

Subfamily: Amphiprioninae

Genus: Amphiprion

The false percula clownfish, *Amphiprion ocellaris*, is a small tropical marine fish indigenous to the Indo-West Pacific. It is a charismatic coral reef fish member of the damselfish family (Pomacentridae), well known by the mutualistic interaction they maintain with sea anemones. They are characterized by particular life history traits, such as complex social structures and mating systems involving sequential hermaphroditism.

Their biology is of interest in ecological and physiological studies and thus these aspects are well documented. The subfamily Amphiprioninae, with 28 species described, is one of the best studied group of marine fish.

The false percula clownfish is a very popular aquarium fish and one of the few marine organisms successfully bred in captivity. It has the advantage of being a robust fish and with its small size it can be easily kept in laboratory conditions. It spawns regularly and its genome is partially available in GenBank (Avella et al., 2009; Olivotto et al., 2010; Marcionetti et al., 2017). With their specific nutritional requirements, typical of marine fish, it represents an valid marine experimental model for nutritional studies.

Oryzias latipes

Class: Actinopterygii

Order: Beloniformes

Family: Adrianichthyidae

Subfamily: Oryziinae

Genus: Oryzias

The Japanese rice fish (*Oryzias latipes*) also known as medaka is a small tropical freshwater and euryhaline fish indigenous to East Asian countries, primarily Japan, Korea, Taiwan, and China. It is an emerging vertebrate model complementary to the zebrafish model in the fields of biomedicine, developmental biology, genetics and toxicology (Kinoshita, M., Murata, K., Naruse, K., & Tanaka, 2009).

Medaka is a small fish (up to 3 cm) that inhabit lowland and brackish waters. The genus Oryzias comes from the Greek “ὄρυζα” (oryza) that means “rice”, in reference to their high abundance in rice paddy fields (Froese and Pauly, 2017). Medaka is a ommnivorous temperate-zone fish and is thus capable of tolerating a wide range of temperatures (10–40 °C). This fish has been a popular aquarium pet in japan for centuries (Kinoshita et al., 2012).

Due to their limited swimming capacity and fragmented distribution, they are among the most diverse vertebrates (Kinoshita et al., 2012). The large genetic divergence among regional populations (3%–4% sequence divergence among regional populations) is not found in other vertebrate models. The evolutionary divergence with zebrafish is also high. The approximate divergence time has being estimated to be 324 million years with zebrafish and, as a comparison, 485 million years with mammals. This evolutionary distance is reflected in many aspects of their biology, including the early development and sex determination (Kinoshita et al., 2012).

Like in zebrafish, advanced tools are widely applied thanks to an extensive amount of information made available by genome and transcriptome sequencing. In addition, all known zebrafish techniques also apply to medaka. Medaka is a cheap and easy-to-keep aquarium fish, with a high fecundity and short generation time (approximately 3 months), thus a potent model for nutritional studies. In addition, due to their high salinity tolerance it can be a model not only for freshwater fish, but at the same time, for marine and brackish water fish, including the commercially important Atlantic salmon.

Hermetia illucens

Class: Insecta

Order: Diptera

Family: Stratiomyidae

Subfamily: Hermetiinae

Genus: Hermetia

The black soldier fly, *Hermetia illucens*, is a detritivorous insect belonging to the order Diptera and the family Stratiomyidae that occurs worldwide in tropical and temperate regions. Its life cycle comprise four advantageous live stages: egg, larvae, pupae, and adult. The adults, which are poor flyers, typically mate during two days, time after which, females lay a single clutch of about 500 to 1200 eggs, depending on the fertility level of the female (Spranghers et al., 2017). Eggs are typically deposited on moist substrate such as decaying vegetable and animal material. Its larvae pass through five instars before

molting into pupae larvae and hatch in 4-6 days at 27°C (Gobbi et al., 2013). During this stage this voracious scavenger feed on a variety of decomposing organic resources such as plants, algae, manure and food waste. The larvae devour from 25 to 500 mg of organic matter per larva per day depending on the larval size, substrate, and environmental factors (e.g., moisture, temperature, and air supply). After completing its larval development, the insect enters the prepupal stage, in which the larva stops feeding and empties its digestive tract. From this stage on larvae and adult does not feed and relies solely on its body fat reserve. The prepupa is highly nutritious with 32%-44% of raw protein and 33%-35% of crude fat (Li et al., 2016; Barragan-Fonseca et al., 2017; Liland et al., 2017; Vargas et al., 2018).

A great advantage for harvesting of this species is that in order to pupate they migrate away from the source of development in search of a dry and protected site and thus the fly avoid to come into contact with any degrading or fresh organic material. Several cage systems have being developed to self-collect pre-pupae in the final stage and to stockpile them prior to processing or other utilization.

BSF is a good source of protein, lipids (mostly C12 fatty acids) vitamins and minerals (rich in calcium manganese, iron and zinc) but it is highly dependent on the developmental stage and the feeding media (Spranghers et al., 2017). Crude proteins varies from 307 to 588.0 g kg^{-1} DM, while crude lipids varies from 113- 407 g kg^{-1} DM. While the largest lipid fraction (typically above 50%) of the insect larvae is undesirable SFAs, a growing number of researchers are reporting new sustainable row materials able to enhance their PUFAs content (Meneguz et al., 2018; Shumo et al., 2019).

Scope of the thesis

The present Ph.D. thesis examined the possible application of innovative feed ingredients and feed additives alternative to fish meal and fish oil as a mean to contribute to the expansion of aquaculture in a sustainable and cost-effective way. In particular, the present thesis focused its attention on the use of fish meal from the black soldier fly (*Hermetia illucens*) as total or partial replacement in marine and fresh water fish models (zebrafish and clownfish) with the aim of generalize the biological responses of the powerful models selected on aquaculture relevant species. In addition, the present thesis explored the use two macroalgae (*Palmaria palmata* and *Saccarina latissima*) as feed additives on the Japanese rice fish (*Oryzias latipes*), Medaka. A comprehensive

knowledge about the physiological responses of the experimental models was thoroughly investigated through multidisciplinary approaches including biometric, histological, spectroscopic, biochemical, and molecular analysis.

In the first chapter, the possible application of a 100% insect diet in zebrafish larval rearing was, for the first time, investigated. To this aim, black soldier fly was produced on industrial wastes such as byproducts obtained from roasting coffee process. Physiological and molecular mechanisms of the fish were analysed and discussed. Results showed that this novel feed was able to sustain fish growth.

In the second chapter, formulated diets with increasing replacement (0%–25%–50%) of fish meal by BSF full-fat prepupae meal were designed and tested on zebrafish larvae. This work contributed to the understanding of the effects of insect meal on the fish lipid metabolism, stress, immune system, growth and the role of chitin in fish nutrition and welfare. Results were promising, with comparable stress, growth and survival levels, but a 50% BSF meal inclusion in the diet affected both lipid composition and hepatic accumulation in the larvae.

In the third chapter, an experiment with the marine fish model *A. ocellaris* was performed using defatted BSF prepupae meal. Formulated diets with increasing replacement levels of insect meal respect to fish meal (0%–25%–50%, 75%) were examined. This was the first study on a marine ornamental fish species. Although the fish fatty acid was strongly affected by the novel ingredient, analysis of the mechanisms involved in the fish growth, lipid metabolism and stress response, allowed to stablish that the insect meal was a good fish meal replacement.

Finally, in the fourth chapter, the effects of seaweed fractions (*Palmaria palmata* and *Saccarina latissima*) as feed additives on the gut structure and immune responses of medaka were analysed. Additionally, the present investigation aimed to establish medaka as a model fish species for nutritional studies, employing histological as well as molecular tools to reveal diet effects. Seaweeds did not affect fish growth and gut morphology, with no sights of inflammation. Indeed, a weak but significant immunomodulatory effect was revealed, supporting the potential of medaka as a novel feed-induced inflammation model.

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Chapter 1

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Rearing zebrafish on black soldier fly (*Hermetia illucens*): biometric, histological, spectroscopic, biochemical and molecular implications.

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Abstract

A desirable goal of the aquaculture sector is to replace most of fish meal and fish oil with more sustainable, cost-effective and environmental friendly ingredients ensuring fish health and welfare standards. Due to minimal environmental impact, compared to most conventional feed commodities, insects deserve a growing attention as candidate ingredients for aquafeeds. The present study investigated, for the first time, the possible application of a 100% insect diet in zebrafish larval rearing. Through a multidisciplinary approach, the major biological responses of fish to the new diets were assessed. Results of biometry, fatty acid composition, expression of genes involved in fish growth, stress response, lipid metabolism, chitinolytic activity, gut inflammation and liver macromolecular composition suggested a possible application insect larvae for zebrafish larval rearing. However, further studies are necessary to better understand the use of this insect species in the rearing of fish.

Keywords

Black soldier fly, gene expression, fatty acid profile, insect diet, aquaculture

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INTRODUCTION

In contrast to world capture fisheries production, which has essentially stagnated since the mid-1980s, aquaculture has maintained an important annual growth rate worldwide since 2005 [1–3]. Fish meal and

fish oil still represent a crucial part of current industrially produced diets, with approximately 20 million tons of total marine catches going into the production of aquafeed [2–4]. Because over-exploitation of pelagic fisheries has negative ecological and social consequences, developing a strategy to replace fish meal and fish oil in feeds should become both a private and public-sector priority in order to reduce pressure and reliance on marine resources while increasing producer profitability [5,6]. Thus, world-renowned nutritionists and feed technologists are exploring practical ways for the aquaculture industry to expand and remain competitive, and discuss ways to develop less expensive alternative sources of lipid and protein.

Research to develop substitutes for these feed ingredients is now focused on commodities such as oilseeds (especially soybeans), meat byproducts (such as blood meal and bone meal) and microbial proteins [6]. A move towards partial substitution of plant and terrestrial animal proteins for fish proteins in feed is widely accepted within the aquaculture industry, but the urgency of such efforts remains controversial. Nevertheless, complete replacement of fish meal and fish oil in aquaculture feeds faces severe barriers [4]. Especially for carnivorous fish, vegetable proteins have inappropriate amino-acid balance, poor protein digestibility and antinutritional substances [7]. Therefore, inclusion of microalgae [8,9] and/or meat by-products can help in overcome this problem.

Recently, a great interest has arisen for the use of insects as a source for feed production [10], especially because of their animal origin, their ability of growing on a great variety of organic compounds, including organic wastes, and to the fact that their consumption in animal farming is not in direct competition with human nutrition [11–14]. Culturing systems are usually simple and require low cost materials; however, insects nutritional profiles largely depend on the growing substrate but also on the selected species and stages of development [15–17], underlying the need of further studies to better understand their possible application in the aquaculture field. For example, the study by Barroso and collaborators [16] evidenced a poor n-3 and n-6 fatty acids content in 16 different insect species belonging to Coleoptera, Diptera and Orthoptera order. However, ST-Hilaire and collaborators [18]

showed that the use of fish offal as growing substrate, along with cow manure, enhanced the lipid profile of the black soldier fly (BSF), *Hermetia illucens* (L.) (Diptera, Stratiomyidae), in terms of omega-3 fatty acids.

Insects may be carriers of biological and chemical hazards, such as prions, viruses, parasites, organic contaminants (e.g. dioxin compounds and pesticides) and heavy metals [19,20]. Proper management and prevention during insect production is therefore a must; in fact, a recent study suggested that preventive techniques similar to that of other farmed animals should guarantee food safety of edible insects [20].

Insects are characterized by having an exoskeleton of chitin. Fish have limited digestive capacity for chitin and it has been demonstrated that diets rich in chitin can have a negative impact on food intake, availability, digestibility and absorption of nutrients and thus growth performances [21].

Among the different insect species that have been cultured and studied for their possible application in the aquaculture field, BSF represents one of the most interesting ones [18,22]. Rearing BSF has been proposed since the 1990s as an efficient way for converting organic wastes into a protein-rich and fat-rich biomass suitable for various purposes, including animal feeding [23]. It is an extremely resistant species and the larvae process organic wastes very quickly [23]. The adult does not feed and therefore does not require particular care and is not a potential carrier of diseases [23]. However, its culture requires a warm environment, which may be difficult or energy-consuming to sustain in temperate climates. Nowadays, feeds based on BSF larvae open additional marketing opportunities in the aquaculture sector [10].

Future growth and profitability within the aquaculture sector is dependent upon continued improvements in diet efficiency and formulation; specifically a reduction or even a complete substitution of unsustainable marine protein and lipid feedstuff is desirable and insects may thus represent an ideal alternative. In line with this, the aim of the present study was to evaluate the

suitability of a 100% insect diet, based on BSF grown on two different substrates, on zebrafish larval development through a multidisciplinary approach including morphological, biochemical, spectroscopic and molecular analysis. Zebrafish represents an extraordinary experimental model for biomedical, developmental biology, genetics, toxicology and aquaculture studies, due to its high reproductive rate and to the abundant information that has recently become available from genomic sequencing [24,25]. Particularly, zebrafish are used to generalize how several biological processes occur in related organisms, and contribute toward improving our understanding of the mechanisms involved in nutrition and growth [26–29].

MATERIAL AND 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. 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 anesthetic (MS222; Sigma Aldrich).

Insect sources

Significant amounts of BSF were cultured at different conditions as follows:

Group A: BSF were reared on by-products obtained from roasting coffee process [provided by Saccaria Caffè S.R.L., Marina di Montemarciano (AN), Italy]. BSF laboratory colony was originated from commercially available prepupae [Smart Bugs s.s. company, Ponzano Veneto (TV), Italy]. The colony was maintained under controlled conditions (30 ± 2°C, 70 ± 10% RH and 14:10 L:D). Prepupae were placed in different plastic boxes (57 x 39 x 28 cm /45 L) until pupa stage was reached. Emerged adults were placed in 2 m^3 cages where the mating occurred. Coffee by-products were used as egg-laying substrate. Three thousand 4 day old larvae were transferred in each of the plastic boxes

containing coffee substrate and feed was supplied ad libitum until larvae reached the post-feeding stage of development, indicated by their change in color from cream to black [30]. The life cycle, from egg to prepupae, occurred approximately in 4 months. Prepupae were collected with a ramp and a collection device as they migrated away from the feeding substrate to pupate.

Group B: BSF prepupae were purchased from a commercial company [Smart Bugs s.s. company, Ponzano Veneto (TV), Italy]. Insects were fed on a feeding substrate composed by corn meal and fruit and vegetable mixture (50:50).

All prepupae (group A and group B), once collected, were frozen (at -80 °C), freeze-dried, minced and sieved (300 μm).

Fish feeding trial

Zebrafish AB embryos were spawned and maintained 48 h in a Tecniplast system (Varese, Italy), subjected to the following conditions: pH 7; NO_2 and NH_3 < 0.01 mg L^{-1}; $N0_3$ < 10 mg L^{-1}, photoperiod 12L: 12D. After this first period, embryos were gently collected, counted under a stereomicroscope (Leica Wild M3B, Leica Microsystems) and divided in nine experimental tanks (200 larvae each in 20 L tanks. The water in the 20 L larval tank (same chemical-physical characteristics of the parent's tank) was gently replaced ten times a day by a dripping system. The sides of the tank were covered with black panels to reduce light reflection [31,32].) that were randomly assigned to the following three dietary treatments (each in triplicate):

Control group: larvae fed a commercial feed (Blue Line, Italy; for composition http://www.blue-co.it/alimenti-in-pellets.html); group A: larvae fed the BSF grown on by-products obtained from roasting coffee process (Proximate composition %: Crude protein: 34.7± 2.2 ; Ether Extract: 18.8 ± 0.5 ; Ash: 10.0 ± 0.4; Nitrogen Free extract: 36.4 ± 4.2); group B: larvae fed on the BSF grown on corn meal, fruit and vegetable mixture (50:50) (Proximate composition %: Crude protein: 36.7± 4.2; Ether Extract: 16.8 ± 0.8 ; Ash 11.8 ± 0.3; Nitrogen Free extract: 34.6 ± 3.2)All groups

were fed on the rotifer *Brachionus plicatilis* (5 ind/mL) from 5 to 10 days post spawning (dps; one feeding in the morning) according to Lawrence and collaborators [33]. Zebrafish larvae were maintained in 20 L tanks and fed the experimental diets at 2% body weight, twice a day [24,25]. Larvae were sampled at 7, 14 and 21 dps, euthanized with an excess of anesthetic (MS222 1g/L, Sigma) and properly stored for further analysis.

Biometry and survival

At each sampling time, ten zebrafish larvae, in triplicate, were randomly collected from the different tanks. The standard length was estimated through the use of a caliper (Measy, New Zealand) (precision 0.1 mm) and the dry weight (calculated in pools of ten larvae). Larvae were rinsed with fresh distilled water, placed in individual, pre-weighed capsules, dried at 60°C for at least 24 h and finally weighed (Mettler-Toledo microgram balance UMX2 automated-s ultra-microbalance, (Columbus, OH, USA) and UM3 precision single pan balance, Switzerland). The specific growth rate (SGR) for each experimental group/ tank was calculated as follows:

$$SGR\% = ((\ln Wf - \ln Wi)/t) \times 100,$$

where Wf is the final dry weight (21 dps), Wi, the initial dry weight (7 dps), and t, the number of days (14). Finally, survival was determined by counting the dead and alive larvae (in triplicate) at the end of the experiment (21 dps).

Lipid extraction and fatty acid analysis

BSF feeding substrates (group A and group B), experimental diets (control group, group A and group B), and larval fish samples collected at 21 dps (control group, group A and group B), each in triplicate, were minced, homogenized (MZ 4110, DCG Eletronic) and freeze-dried (Edwards EF4, Crawley, Sussex, England). Lipid extraction was carried out on lyophilized powders following a Microwave-Assisted Extraction (MAE) [34,35]. Fatty acid methyl esters (FAMEs) were prepared according to Truzzi and collaborators (2017), using the methyl ester of nonadecanoic acid (19:0, Dr. Ehrenstorfer GmbH,

Germany) as internal standard. FAMEs were determined on an Agilent-6890 GC System coupled to an Agilent-5973N quadrupole Mass Selective Detector (MSD). A CPS ANALITICA CC-wax-MS (30 m × 0.25 mm ID, 0.25 μm film thickness) capillary column was used to separate FAMEs. Instrumental conditions were set up as in Truzzi and collaborators [34], and no overlapping between peaks were noted. The mass fraction of fatty acids as mg g^{-1} tissue dry weight (dw) was measured against internal standard. For each sample, at least three runs were performed on the GC-MS.

The precision of the proposed method was evaluated for the studied matrices as in Truzzi and collaborators [36], and it was similar to that found for fish muscle [34]. For insect substrates, experimental diets and zebrafish larvae, the intra-day and inter-day precision were, for major FAs, < 4% and < 9%, respectively, indicating a good repeatability of the analyses (data not shown). For FAs with a percentage <1% *vs* total FAs, intra-day and inter-day precision ranged from 6% to 20%, and from 7 to 25%, respectively. The estimated limits of detection (LOD) and limits of quantification (LOQ), calculated as in Truzzi and collaborators [37], ranged for each FAME from ~4 μg mL^{-1} to ~22 μg mL^{-1}, and from ~13 μg mL^{-1} to ~66 μg mL^{-1}, respectively [34].

Histology

Ten zebrafish larvae, in triplicate, randomly collected at each sampling time from the different tanks, were fixed by immersion in 4 % paraformaldehyde and stored at 4 °C for 24 h. Larvae were washed 3 times with PBS 0.1 M (pH 7.4) buffer for ten minutes and preserved in ethanol (70 %). Larvae were then dehydrated by subsequent washing in ethanol (80, 95 and 100 %), washed with clearing agent "Histo-Clear" (Bio-Clear, Bio Optica) and embedded in liquid paraffin (Bio-Optica, Milano, Italy) at 55-58 °C. Solidified paraffin blocks were cut with a microtome (Leica RM2125 RTS) in order to obtain 5 μm sections then stained with hematoxylin (Mayer) and eosin Y (Sigma-Aldrich). Sections were observed using a Leica MD750 optical microscope connected with a camera Leica ICC50 HD.

Fourier Transform Infrared Microspectroscopy (FTIRM) measurements and data analysis

Intestines from five different specimens of 21 dps zebrafish larvae fed BSF diet (group A and group B) and a commercial feed (control group), were quickly dissected and immediately frozen at -80°C. Then, each intestine sample was cut by using a cryomicrotome (Microm HM 505 N) to obtain six thin adjacent sections (~10 μm thick): three sections were deposited onto CaF_2 optical windows (1 mm thick, 13 mm diameter) for FTIRM analysis, while the other three sections were placed onto conventional glass slides for morphological examination (haematoxylin and eosin staining, H&E) [38].

FTIR Microspectroscopy studies the interaction between the infrared radiation and the chemical bonds and couples the diagnostic potential of IR spectroscopy with the visual inspection performed by Vis microscopy. It let analyze non-homogeneous tissue sections by acquiring on the same sample and at the same time the microphotograph and the IR map, which represents the distribution of the total absorption of the infrared radiation on the mapped area. For each pixel of the map, it is possible to retrieve, the corresponding vibrational signature, and hence the macromolecular building and composition of the sample [39,40].

FTIRM analysis was performed at room temperature on 45 sections (15 for each experimental group) within 24 hours from cutting. Intestine sections prepared by using this protocol have been already tested in previous experiments in our laboratory, evidencing a good stability in time and providing homogeneous and reliable vibrational data sets. A Perkin Elmer Spectrum GXI Spectrometer, equipped with a Perkin Elmer Autoimage microscope and a photoconductive HgCdTe MCT array detector, operating at liquid nitrogen temperature, which covers the entire IR spectral range from 4000 to 700 cm^{-1}, was used.

By means of a microscope television camera, specific areas were selected on each section, according with the histological suggestions. On these areas, the IR maps (~300 x 500 μm^2), were acquired in transmission mode in the MIR range from 4000 cm^{-1} to 800 cm^{-1} (spectral resolution 4 cm^{-1}, spatial resolution 40x40 μm^2 and 128 scans). Background spectra were acquired on clean portions of the

CaF_2 optical windows and compared against samples spectra. IR maps are false colour images representing the total intensity of the infrared absorption; each pixel, whose dimensions correspond to the spatial resolution, is represented by a single IR spectrum. IR maps were automatically corrected for avoiding the contributions of carbon dioxide and water vapour and vector normalized on the full frequency range (to avoid artifacts due to local thickness variations). Spectrum Image 5.1.0 (Perkin Elmer) and Spectrum 10.4 (Perkin Elmer) software were used for data handling.

From each map, 50 spectra were selected and vector normalized. Then, the area integrals of the following spectral regions were calculated: 3000-2815 cm^{-1} (corresponding to the symmetric and asymmetric stretching modes of CH_2 and CH_3 groups, representative of lipids, named LIP); 1771-1715 cm^{-1} (corresponding to the stretching mode of C=O group of fatty acids, named FA); 1715-1481 cm^{-1} (corresponding to Amide I and Amide II bands of proteins, named PRT); 1481-1425 cm^{-1} (corresponding to CH_2 groups of alkyl chains, named CH2), and 1143-985 cm^{-1} (corresponding to glycosylated proteins, and mainly mucin, named MUCIN). The area integrals of the 2998-2800 cm^{-1} and 1775-950 cm^{-1} were added, and their sum was considered representative of the total tissue biomass (TBM). The aforementioned integrals were used to calculate the following band area ratios: LIP/TBM, FA/TBM, CH2/TBM, PRT/TBM, and MUCIN/TBM.

RNA extraction and cDNA synthesis

Total RNA extraction from 20 zebrafish larvae (in triplicate), randomly collected from the different tanks at each sampling point, was optimized using RNAzol® RT reagent (SIGMA-ALDRICH®, R4533) following the manufacturer's instructions. RNA was then eluted in 20 µl of RNase-free water. Final RNA concentration was determined by the NanoPhotometer® P-Class (Implen, 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, 3 µg of RNA was used for cDNA synthesis, employing the High Capacity cDNA Reverse Transcription Kit (Bio-Rad) following the manufacturer's instructions.

Real-Time PCR

PCRs were performed with SYBER Green in an iQ5 iCycler thermal cycler (both from Bio-Rad) in triplicate. Reactions were set on a 96-well plate by mixing for each sample 1 µL cDNA diluted 1:20, 5 µL of 2x concentrated iQ^{TM} Syber Green as the fluorescent intercalating agent, 0.3 µM forward primer, and 0.3 µM 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 60 °C and 20 s at 72 °C. Fluorescence was monitored at the end of each cycle. Dissociation curve analysis showed a single pick in all cases.

Relative quantification of the expression of genes involved in fish growth (*igf1*, *igf2a* and *mstnb*), fatty acid desaturation (*fad2*) and elongation (*elovl5* and *elovl2*), stress response (*nr3c1*and *hsp70.1*) and enzymatic hydrolysis of chitin (*chia.1, chia.2, chia.3, chia.4, chia.5* and *chia.6*) was performed using *rplp0* and *rpl13* as housekeeping genes to standardize the results (Table 1).

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 analyzed 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 is reported with respect to controls. Primer sequences were designed using Primer3 (210 v. 0.4.0) starting from zebrafish sequences available in ZFIN. Primers were used at a final concentration of 10 pmol/µL.

Statistics

Survival, SGR and FTIRM data were analyzed by one way ANOVA, with diet as the explanatory variable. Lipid content and fatty acid data were analyzed by both t-test and one-way ANOVA. Standard length, dry weight and real time PCR data were analyzed by two-way ANOVA, with both diet and dps as the explanatory variables. Normality and homogeneity of variance was verified

through residual plots according to Zuur and collaborators [41], revealing no violations of ANOVA and t-test assumptions. All ANOVA tests were followed by Tukey's post-test. The statistical software package Prism5 (GraphPad Software) was used; significance was set at $P < 0.05$. All results are presented as mean ± S.D.

RESULTS

Biometry

At the end of the experiment (21 dps), the survival of the zebrafish larvae did not exhibit significant statistical differences among the dietary treatments ($P > 0.05$), with control group showing the highest value (44 ± 14 %) with respect to group A and B (38 ± 20 % and 30 ± 5 %, respectively) (Table 2).

As regards the standard length, no significant differences ($P > 0.05$) among the experimental groups were detected at all sampling times (7, 14 and 21 dps; Table 2). At the end of the experiment, control group larvae showed the highest standard length (6.5 ± 1.2 mm), while group A and group B showed lower standard length with respect to control group (5.7 ± 0.8 mm and 5.4 ± 1.1 mm, respectively; Table 2). As regards dry weight, no significant differences ($P > 0.05$) were observed among the three experimental groups, at all experimental times (7, 14, 21 dps; Table 2). At 21 dps control group larvae showed the highest dry weight (4.5 ± 2.3 mg), while group A and group B showed a 2.3 ± 0.8 mg and 1.8 ± 0.7 mg dry weight, respectively (Table 2). However, no statistical differences were observed ($P > 0.05$). Finally, considering SGR, control group larvae showed the highest value (27.1 ± 3.5), while group A and group B showed a 22.4 ± 2.6 and 19.5 ± 4.6 SGR, respectively (Table 2); no significant differences were detected ($P > 0.05$).

Lipid content

Table 3 shows the dry weight (dw) and lipid percentage of BSF feeding substrates, experimental diets and zebrafish larvae collected at 21 dps. Group A substrate showed dry weight significantly lower ($P < 0.05$) than group B substrate (52.3 ± 0.9 and 89.6 ± 0.4, respectively). Lipid percentage of substrates

showed no significant differences (group A 3.4 ± 0.1 % and group B 3.0 ± 0.7 %; $P > 0.05$). As regards the diets, both control group and group A, showed a significantly ($P < 0.05$) higher dry weight (97.6 ± 0.2 mg and 98.6 ± 0.3 mg, respectively) with respect to group B (92.4 ± 0.6 mg). Lipid percentage of control group and group A diet showed no significant differences (11 ± 1 % and 13 ± 1 %, respectively; $P > 0.05$), while group B diet showed a significantly higher lipid percentage (25 ± 1 %; $P < 0.05$) with respect to both control group and group A. Considering zebrafish larvae, group A samples (23.6 ± 3.2 %) did not show significant differences ($P > 0.05$) in dw percentage with respect to control group (16.7 ± 2.7 %). Group B larvae (15.9 ± 3.0 %) showed a dry weight percentage significantly lower ($P < 0.05$) than group A. As regards the lipid percentage of the larvae, no significant differences ($P > 0.05$) among experimental groups were observed (control group 9.5 ± 1.8, group A 7.3 ± 3.2 and group B 12.7 ± 3.8 %; Table 3).

Fatty acids composition

Table 4 reports the FAs composition (as mg g^{-1} dw) of the insect substrates, the experimental diets and zebrafish larvae collected at 21 dps.

Both insect substrates were characterized by a high content of palmitic acid (16:0) that, with stearic acid (18:0), represented more than 70% of SFA. Among MUFA, oleic acid (18:1n9) was the most important; PUFA were not present, with the exception of linoleic acid (18:2n6) and α-linolenic acid (18:3n3).

As regards the diets, group A and group B showed a very high content of lauric acid (12:0) that represented about 55% of SFA, and ~40% of the total FAs, for both groups. On the contrary, SFA of control group diet were characterized by a high content of palmitic acid (16:0), followed by stearic acid (18:0) and myristic acid (14:0). The most important MUFA were oleic acid (18:1n9) for all experimental diets; palmitoleic acid (16:1n7) was also present in significant quantity. Control group diet showed a high content of omega-3 EPA (eicosa-5,8,11,14,17-pentaenoic acid, 20:5n3), DHA

(docosa-4,7,10,13,16,19-hexaenoic acid, 22:6n3), and linoleic acid (18:2n6), whereas group A and group B showed a high content of linoleic acid (18:2n6) but did not show PUFA with C number > 18, by consequence they were poor in n3 FAs.

The control group of zebrafish larvae showed a high content of palmitic acid (16:0), followed by stearic and myristic acids (14:0); in addition group A and group B also showed a high content of lauric acid (12:0). The most represented MUFA were the same detected in the diets, with oleic acid showing the highest content, followed by palmitoleic acid and vaccenic acid (18:1n7). About PUFA, control group showed high content of EPA, DHA, and linoleic acid (18:2n6); group A and B showed a high content of linoleic acid and they also showed a significant ($P < 0.05$) content of DHA.

Figure 1 reports the composition (as mg g^{-1} dw) of saturated (SFA), monounsaturated (MUFA), and polyunsaturated (PUFA) fatty acids, as well as the omega 3 (n3), omega 6 (n6) and omega 9 (n9) content, and the n6/n3 ratio in the insect substrates (Fig. 1a), the experimental diets (Fig. 1b) and zebrafish larvae collected at 21 dps (Fig. 1c).

Substrates- Group A substrate was characterized by a remarkably higher concentration of SFA ($P < 0.05$; 40.7 ±13.9 mg g^{-1} dw) and a lower PUFA content ($P < 0.05$; 0.7 ± 0.1 mg g^{-1} dw) compared to group B substrate (SFA 1.3 ± 0.3 mg g^{-1} dw, PUFA 2.8 ± 0.7 mg g^{-1} dw, respectively; Fig. 1a). Group A substrate showed significantly ($P < 0.05$) less n6 (0.7 ± 0.1 mg g^{-1} dw) with respect to group B substrate (2.3 ± 0.5 mg g^{-1} dw) and a higher content of n9 (3.7 ± 1.3 mg g^{-1} dw) with respect to group B substrate (0.9 ± 0.2 mg g^{-1} dw). Unlike group B, group A did not show n3 FAs. As regards the n6/n3 ratio, no significant differences ($P < 0.05$) between substrates were observed.

Diets- Both group A and group B diets showed a significantly ($P < 0.05$) higher SFA content (50.1 ± 2.8 and 80.2 ± 6.2 mg g^{-1} dw, respectively) and a significantly ($P < 0.05$) lower MUFA content (16.0 ± 0.4 mg g^{-1} dw and 21.9 ± 0.8 mg g^{-1} dw, respectively) with respect to control group (SFA 29.3 ± 1.4 mg g^{-1} dw and MUFA 24.9 ± 1.2 mg g^{-1} dw). Regarding the PUFA content, both group A and

group B diets showed a significantly ($P < 0.05$) lower content (4.6 ± 0.1 and 8.7 ± 0.3 mg g^{-1} dw, respectively) with respect to control group (50.8 ± 1.5 mg g^{-1} dw; Fig. 1b).

Considering FAs classes, the contribution of n6, n3, and n9 on lipid profile significantly varied among the three diets ($P < 0.05$). The n6 content in group A (4.2 ± 0.1 mg g^{-1} dw) and in group B (7.8 ± 0.3 mg g^{-1} dw) showed a significantly lower ($P < 0.05$) value with respect control group (13.7 ± 0.9 mg g^{-1} dw). The n3 content in both group A (0.50 ± 0.01 mg g^{-1} dw) and group B (1.00 ± 0.03 mg g^{-1} dw) was significantly ($P < 0.05$) lower with respect to control group (37.1 ± 1.2 mg g^{-1} dw). Consequently, the n6/n3 ratio was significantly higher ($P < 0.05$) in both group A (8.8 ± 0.3 mg g^{-1} dw) and group B (8.2 ± 0.4 mg g^{-1} dw) with respect to control group (0.37 ± 0.03 mg g^{-1} dw) diet. Finally, the n9 FA content in group A diet (8.6 ± 0.2 mg g^{-1} dw) was significantly lower ($P < 0.05$) with respect to both control group (17.2 ± 1.0 mg g^{-1} dw) and group B (15.6 ± 0.6 mg g^{-1} dw) diets.

Zebrafish Larvae- Group A did not show significant ($P > 0.05$) differences in SFA (33 ± 2 mg g^{-1} dw) and MUFA content (18.2 ± 0.9 mg g^{-1} dw) with respect to control group (SFA 28.7 ± 2.8 mg g^{-1} dw and MUFA 19.8 ± 1.1 mg g^{-1} dw), whereas group B showed a significantly higher content (SFA 71.8 ± 5.4 mg g^{-1} dw and MUFA 27.2 ± 0.8 mg g^{-1} dw; $P < 0.05$) with respect to both control group and group A (Fig. 1c). Regarding PUFAs, both group A and B larvae showed a significantly lower ($P < 0.05$) content (10.8 ± 1.1 and 16.2 ± 1.5 mg g^{-1} dw, respectively) with respect to control group (34.5 ± 1.6 mg g^{-1} dw); group A showed significantly lower ($P < 0.05$) PUFA content with respect to group B.

Considering the n6 content, group A larvae showed a significantly lower ($P < 0.05$) n6 content (5.3 ± 0.6 mg g^{-1} dw) with respect to both control group and group B (11.3 ± 0.8 and 10.8 ± 0.4 mg g^{-1} dw, respectively). As regards the n3 content, both group A and B larvae showed a significantly lower ($P < 0.05$) n3 content (5.5 ± 0.9 mg g^{-1} dw and 5.4 ± 1.4 mg g^{-1} dw, respectively) with respect to control group (23.2 ± 1.5 mg g^{-1} dw; Fig. 1c). Consequently, the n6/n3 ratio in group A (1.0 ± 0.1 mg g^{-1} dw) did not show significant differences ($P > 0.05$) with respect to control group (0.5 ± 0.04 mg

g^{-1} dw), whereas group B (2.0 ± 0.5 mg g^{-1} dw) showed a significantly higher value ($P < 0.05$) with respect to both group A and control group. Finally, as regards the n9 content, group A (11.3 ± 0.5 mg g^{-1} dw) showed significantly lower values ($P < 0.05$) with respect to control group (14.1 ± 0.9 mg g^{-1} dw), while group B (17.4 ± 0.7 mg g^{-1} dw) showed a significantly higher ($P < 0.05$) value with respect to both control group and group A.

Histological and infrared analysis

No appreciable differences among larvae fed BSF diets (group A and group B) and a commercial feed (control group) were detected at 7 and 14 dps in the intestinal mucosa of all analyzed samples (Fig. 2a-f). A general increase in goblet cell number was evident at 21 dps only in group B samples with respect to both control group and group A (Fig. 2g-i). In addition, no severe histological alterations were thus observed in all experimental groups in terms of inflammatory influx and morphological alterations of intestinal folds.

For a deeper insight of the aforementioned modifications, the FTIRM analysis of intestine sections from 21 dps zebrafish larvae fed BSF meals (group A and group B) and a commercial meal (control group) was performed. By the analysis of the IR spectra of each experimental group, the following absorption bands were detected: 3016 cm^{-1} (=CH stretching mode of lipid alkyl chains) [42]; 2960 cm^{-1}, 2873 cm^{-1} (CH_3 asymmetric and symmetric stretching modes of lipid alkyl chains, $\nu_{asym}CH_3$ and $\nu_{sym}CH_3$), and 2926 cm^{-1}, 2850 cm^{-1} (CH_2 asymmetric and symmetric stretching modes of lipid alkyl chains, $\nu_{asym}CH_2$ and $\nu_{sym}CH_2$) [42]; 1747 cm^{-1} (vibrational modes of fatty acids) [43]; 1653 cm^{-1} and 1546 cm^{-1} (Amide I and Amide II bands of proteins, attributable to C=O and C-N stretching, and N-H bending modes) [44]; 1455 cm^{-1} (CH_2 bending modes of alkyl chains) [45]; 1399 cm^{-1} (COO- carboxylate groups of aspartate and glutamate amino acids) [45,46]; 1241 cm^{-1} (collagen vibrational modes) [47]; 1143 cm^{-1} (C-OH stretching vibrations of carbohydrates) [45], and 1100 cm^{-1} and 1040 cm^{-1} (glycosylated proteins) [48].

A semi quantitative analysis was performed on the spectra extracted from IR maps. For this purpose, the following band area ratios were calculated: LIP/TBM (representative of total lipids); FA/TBM (representative of total fatty acids); CH2/TBM (representative of alkyl chains length); PRT/TBM (representative of total proteins), and MUCIN/TBM (representative of total mucin). Their variation among the experimental groups was analyzed. In group A and group B, a statistically significant increase ($P < 0.05$) of lipids (LIP/TBM, 0.24 ± 0.02 and 0.38 ± 0.02, respectively; fig. 3e) and in particular of fatty acids (FA/TBM, 0.20 ± 0.10 and 0.25 ± 0.05; fig. 3b) with longer alkyl chains (CH2/TBM2, 0.04 ± 0.00 and 0.07 ± 0.01; fig. 3c) was observed with respect to control group (LIP/TBM 0.08 ± 0.02, FA/TBM 0.07 ± 0.01 and CH2/TBM 0.02 ± 0.01; fig. 3a, b, c). Similarly, a significantly higher ($P < 0.05$) proportion of proteins (PRT/TBM) was also detected in group A (0.96 ± 0.11) and group B (1.04 ± 0.15) with respect to control group (0.71 ± 0.09; fig. 3a), above all attributable to mucin (MUCIN/TBM, group A 0.66 ± 0.10, group B 0.76 ± 0.09 and control group 0.19 ± 0.05; fig. 3d).

Concerning the histological analysis of the liver, group A samples did not show any alteration at the hepatocytes level during the whole experimental period (Fig. 4b, e, h), with a general aspect comparable to the control group (Fig. 4a, d, g). In group B samples, no morphological alterations were observed with respect to control group till 7dps (Fig. 4c), however, at 14 and 21 dps a general hepatic steatosis, with a conspicuous intracellular accumulation of lipids was observed (Fig. 4f, i).

Real-Time PCR results

Real-time PCR analysis was performed on genes involved in fish growth (*igf1*, *igf2a* and *mstnb*), fatty acid desaturation (*fad2*) and elongation (*elovl5* and *elovl2*), stress response (*nr3c1*and *hsp70.1*) and enzymatic hydrolysis of chitin (*chia.1, chia.2, chia.3, chia.4, chia.5* and *chia.6*).

Growth factors. Regarding *igf1*, a time-dependent increase in gene expression was evident in all experimental groups, with no significant differences ($P > 0.05$) among them (Fig. 5a). A similar result was observed for *igf2a* gene expression, with the exception of group B samples, which showed a

significantly lower ($P < 0.05$) gene expression at 14 dps and a significantly higher ($P < 0.05$) gene expression at 21 dps with respect to both control group and group A (Fig. 5b). Finally, as regards *mstnb* gene expression, results did not show a similar trend to *igfs*. Particularly, group A samples did not show significant differences ($P > 0.05$) with respect to control group at 7 and 14 dps, while a significant downregulation ($P < 0.05$) was observed at 21 dps (Fig. 5c). Group B samples showed a significantly higher ($P < 0.05$) *mstnb* gene expression at 7 dps with respect to both control group and group A while at 14 dps a significant downregulation ($P < 0.05$) in its gene expression was observed. Finally, at 21 dps, a significantly lower ($P < 0.05$) gene expression with respect only to control group was evidenced.

Markers of long-chain polyunsaturated fatty acid biosynthesis. As regards the genes involved in the long-chain polyunsaturated fatty acid biosynthesis, results showed that all desaturases and elongases, in both group A and B, were principally expressed at 21 dps. Particularly, as mentioned above, *fad2* gene expression, in both group A and B samples did not show significant differences ($P > 0.05$) with respect to control group at 7 and 14 dps, whereas at 21 dps both group A and B showed a significant upregulation ($P < 0.05$) with respect to control group (Fig. 6a). A similar result was observed for *elovl5* gene expression (Fig. 6b). Finally, as concerns *elovl2* gene expression, group A samples did not show significant differences ($P > 0.05$) with respect to control group at 7 and 14 dps, whereas at 21 dps a significant upregulation ($P < 0.05$) with respect to control group was evident (Fig. 6c). Group B samples, at 7 dps, did not show any significant difference ($P > 0.05$) in its *elovl2* gene expression with respect to control group, while at 14 dps a significantly higher ($P < 0.05$) gene expression in group B samples with respect to group A was observed (Fig. 6c). Finally, at 21 dps, a significantly higher ($P < 0.05$) gene expression was detected only with respect to control group, while a significantly lower ($P < 0.05$) gene expression was observed with respect to group A (Fig. 6c).

Stress markers. As regards *nr3c1* gene expression, group A samples did not show significant differences ($P > 0.05$) with respect to control group during the whole experimental period (Fig. 7a).

On the contrary, group B samples showed a significantly higher ($P < 0.05$) gene expression only at 7 dps with respect to both control group and group A. Finally at 21 dps, a significantly lower ($P < 0.05$) gene expression in group B samples, only with respect to group A, was detected.

As concerns *hsp70.1* gene expression, group A samples did not show any significant difference ($P > 0.05$) with respect to control group during the whole experimental period (Fig. 7b). Group B samples showed a significantly lower ($P < 0.05$) gene expression at 7 dps with respect to both control group and group A, whereas at 14 dps a significantly higher ($P < 0.05$) gene expression was observed. Finally, at 21 dps no significant differences ($P > 0.05$) among experimental groups were observed.

Markers of enzymatic hydrolysis of chitin. As concerns the enzymatic hydrolysis of chitin, in general, all six genes analyzed showed a time-dependent increase (Fig. 8). Particularly, *chia.1,2,3,4,5* and *6,* gene expression in group A samples did not show any significant difference ($P > 0.05$) during the whole experimental period with respect to control group except for *chia.4* at 21 dps, at which a significantly lower ($P < 0.05$) gene expression was detected (Fig. 8a, b, c, d, e, f). Considering group B samples, a more heterogeneous trend was detected with respect to group A.

Particularly, as regards *chia.1*, no significant differences ($P > 0.05$) at 7 dps among experimental groups were detected, whereas at 14 dps a significantly higher ($P < 0.05$) gene expression, with respect to both control group and group A, was observed in group B (Fig. 8a). Finally at 21 dps, group B did not show any significant difference ($P > 0.05$) among experimental groups (Fig. 8a). As regards *chia.2* gene expression, group B samples showed a significantly lower ($P < 0.05$) gene expression at 7 dps with respect to both control group and group A, whereas at 14 dps no significant differences ($P > 0.05$) among experimental groups were observed (Fig. 8b). Finally at 21 dps, group B samples showed a significantly higher ($P < 0.05$) gene expression only with respect to control group. As regards *chia.3* and *chia.4* gene expression, no significant differences ($P > 0.05$) among experimental groups during the whole experimental period were observed, with the exception of group A samples, which showed a significantly lower ($P < 0.05$) *chia. 4* gene expression at 21 dps with respect to control

group (Fig. 8c, d). As concerns *chia.5* gene expression, group B samples showed a significantly higher ($P < 0.05$) gene expression at 7 dps with respect to both control group and group A, whereas at 14 dps no significant differences ($P > 0.05$) among experimental groups were observed (Fig. 8e). Finally at 21 dps, a significantly lower ($P < 0.05$) gene expression in group B samples, with respect to the other experimental groups, was observed (Fig. 8e). As regards *chia.6* gene expression, Group B samples showed a significantly lower ($P < 0.05$) gene expression at 7 dps only with respect to Group A (Fig. 8f). At 14 dps, group B samples showed a significantly higher ($P < 0.05$) gene expression with respect to control group, whereas at 21 dps no significant differences ($P > 0.05$) among experimental groups were observed.

DISCUSSION

A desirable goal of the aquaculture sector is to replace most of fish meal and fish oil with more sustainable, cost-effective and environmentally friendly ingredients. The search for these alternatives represents a major challenge, since alternatives must ensure fish health and welfare standards by providing proper levels of essential amino acids and PUFAs, high nutrient and energy bioavailability, reduced antinutritional factors [4,6,7]. Due to minimal environmental impact, compared to most conventional feed commodities, insects deserve a growing attention as candidate ingredients in aquafeeds [10,14,49]. Insect meal meets macronutrient requirements of many terrestrial animals and fish; former studies showed suitability of this novel ingredient in diets for swine and broiler chickens as well as in nutrition of rainbow trout (*Oncorhynchus mykiss*), or in channel catfish (*Ictalurus punctatus*) and blue tilapia (*Tilapia aurea*) [18,50–54].

Even though zebrafish is a widely used model organism, information about its dietary preferences is limited and its nutritional requirements are mostly unknown [55]. Wild zebrafish are known to feed on a wide variety of benthic and planktonic crustaceans, worms and insect larvae [56]. McClure and collaborators [57] and Spence and collaborators [58] analysed gut contents of zebrafish sampled in the wild and found that insects, mostly of terrestrial origin, were the predominant prey. Information

obtained through the present study may thus be used to generalize how several biological processes occur in related organisms, and contribute toward improving our understanding of the mechanisms involved in fish nutrition and growth.

Presently, most of the studies performed on fish meal and fish oil replacement with insect meal in aquaculture, used levels of inclusion between 10 and 50% [21,59,60]. At this regard, the present study investigated, for the first time, the possible application of a 100% insect diet in zebrafish larval rearing. In this sense, the economic advantage of the present feeds is recognizable, however, the main scope of the present study was to analyse, through a multidisciplinary approach, the major biological responses of fish to the new diets.

Generally, growth and survival results obtained in the present study demonstrated that fish were not significantly impaired by the new diets. These data were fully supported by the molecular results about growth factors (*igfs* and *msntb*). However, it should be pointed out that different results have been reported for other fish species. For example, Gasco and collaborators [59] reported a reduction in growth in seabream fed increasing amounts of *Tenebrio molitor* (0,5 1,0 g/kg), while Lock and collaborators [61] did not observe negative effects in replacing fish meal with BSF meal in *Salmo salar*. Clearly, results may be dependent on the insect and fish species used, as well as on the fish developmental stage and on the insect nutritional profile.

As one of the major nutrients, dietary lipids play important roles in fish nutrition. At this regard, it is well established that lipids play a pivotal role in fish larval nutrition and are involved as energy source, component of cellular membranes, and absorption of lipophilic nutrients [62]. One important aspect of larval fish nutrition is providing adequate levels of highly unsaturated fatty acids (HUFAs), including EPA and DHA [63]; however, insects are known to be poor in n-3 fatty acids [16,18,64,65].

In the present study, a high SFA content was observed in both insect diets. Group A and B diets showed a significantly higher SFA content (50.1 ± 2.8 and 80.2 ± 6.2 mg g^{-1} dw, respectively) and a

lower PUFA content (4.6 ± 0.1 and 8.7 ± 0.3 mg g^{-1} dw, respectively) with respect to control group diet (SFA 29.3 ± 1.4 mg g^{-1} dw, PUFA 50.8 ± 1.5 mg g^{-1} dw), and they were able to affect fish fatty acid composition.

In fish, it is known that a HUFA and/or PUFA deficiency may stimulate an adaptation mechanism by facilitating a level of bioconversion of C18 to C20 or even to C22 FAs [66,67]. Thus, the differences in the composition of one FA between diets and zebrafish larvae fed these diets could reflect an adaptive mechanism of the fish to equilibrate their FA profile [68]. Zebrafish larvae were able to synthetize longer chain fatty acids starting from shorter precursors; these data are supported by the higher desaturase and elongase gene expression observed in group A and B respect to control group at the end of the experiment and by the GM results.

As already reported, the largest lipid fraction of the insect larvae used in the present study was SFA, of which the majority was represented by the medium-chained fatty acid 12:0 (lauric acid) (27 ± 5 and 44 ± 4 mg g^{-1} dw, group A and B, respectively), which is in line with earlier reports [69]. Particularly, lauric acid, has been reported as a proper energy source for both humans and livestock, as it is efficiently absorbed, digested and β-oxidised (as reviewed by Dayrit [70]). Medium chain fatty acids, mostly C_6–C_{12}, are considered physiologically active compounds which appear to be preferentially utilized as an energy source and reduce the deposition of adipose tissue [71,72]. Senlin and collaborators [73] reported no effects on lipid accumulation in the hepatopancreas and muscle of Jian carp fed insect diets. Similar results have been reported in red drum by Davis and collaborators [74]. However, in the present study, group B larvae showed, at the end of the experiment, hepatic steatosis that was not observed in the other groups. Differences in liver lipid stores among the present and previous studies might be the result of differences in the fish size/species and/or the duration of relevant feeding experiments [75]. In addition group B larvae were fed the fattest diet and this may have a direct effect on lipid steatosis since the amount of lipids provided through the diet was outside the

physiologically tolerable range for zebrafish [76]. This observation is in accordance with FTIRM data which evidenced a higher amount of lipids in A and B groups at intestinal level.

The FTIRM analysis of the intestinal tract of 21 dps zebrafish larvae fed BSF meals (group A and group B), evidenced a significant increase of mucin (MUCIN/TBM; 0.660 ± 0.097 and 0.759 ± 0.086, respectively) with respect to control group (0.195 ± 0.045). A small increment of total proteins (PRT/TBM) was also observed (0.960 ± 0.105 and 1.036 ± 0.150, respectively). This spectroscopic findings were in line with the histological analysis, which evidenced in group B a higher number of mucous cells at intestinal level. These same cells have previously been associated to high lipid diets in mice [77,78]. In addition to compromising metabolic health [76], highly saturated fat diets are known to induce low-grade intestinal inflammation [79]. One of the strategies used to analyse adverse effects on intestine is the evaluation of the number of goblet cells, besides morphological damages, and this is often used as a gold standard for the evaluation of the inflammation degree [80]. In fact mucous counteract inflammation in intestines by shielding the underlined epithelium from exogenous stress. Even if no serious tissue damages were induced in intestine by diet B, an higher degree of mucous cell was observed and considered as a mild inflammatory response to this diet. Finally, as expected, the fatty acid profiles of the larvae, analysed in the present study, generally followed the fatty acid profile of the provided diet. In particular, in 21 dps zebrafish larvae fed BSF meals (group A and group B), FTIRM analysis evidenced an increased in SFA: in fact, the increase of FA/TBM (0.200 ± 0.099 and 0.247 ± 0.045, respectively) and CH2/TBM (0.0372 ± 0.0047 and 0.0678 ± 0.0067, respectively) band area ratios suggested a higher amount of fatty acids with longer saturated alkyl chains.

In addition, medium-chain fatty acids have also been reported to possess antibacterial and antiviral properties and have demonstrated positive effects on gut health [81,82] improving gut health under inflammatory conditions [83]. The exoskeleton of the BSF pre-pupae contains the polysaccharide chitin (approximately 87.0 g kg^{-1}, DM) which might affect the digestibility and the utilization of other

nutrients [84,85]. In addition, high chitin inclusion in aquafeeds may induce intestinal inflammation and a reduction in fish welfare and growth [11,21].

At this regard, in the present study, no drastic signs of intestinal inflammation were observed. A high supplementation of medium-chained fatty acids through the insect diet, possibly avoided the incidence of intestinal mucosa inflammation in fish larvae because of their important role in improving gut health. These data were also supported by molecular analysis on stress markers that did not show significant variations in their expression during the experiment.

Chitin has also been reported to decrease feed intake and growth in carp, tilapia (*Oreochromis niloticus* × *O. aureus*), and Atlantic salmon at inclusion levels of 1% and higher [85–87] while no significant effects were observed in *Oreochromis mossambicus* fed a 60 % insect meal or in eel (*Anguilla japonica*), red sea bream (*Pagrus major*), and yellowtail kingfish (*Seriola quinqueradiata*) fed a 10% chitin supplemented diet [88,89]. The differences observed in the above mentioned species may be explained by the chitinolytic activity of the single fish species. Some species, like turbot, do not have any chitinolytic activity during the early life stages [21], while others, like cobia, showed very high endochitinolytic activity [90]. In cod (*Gadus morhua*) the enzyme chitinase was found in stomach and intestinal tract [91–94], but no chitinolytic bacteria activity was reported. These differences are mainly related to the presence/absence of mechanical structures in fish larvae, able to break down the chitin exoskeleton of preys. Generally, the analysis of the six zebrafish chitinases in larvae fed different diets showed a time-dependent gene expression and pointed out that fish endogenous chitinase gene expression was not related to the amount of chitin provided through the diet. However, this result was not obvious, and opens new hypothesis about a possible involvement of intestinal bacteria in chitin digestion in fish.

Conclusions

The present study opens the possibility for the use of a more sustainable ingredient in the larval rearing of zebrafish. Knowledge obtained using experimental models such as zebrafish are becoming of primary importance for the development of the aquaculture industry. However, further studies are necessary to better understand fish responses and to evaluate possible effects on the different stages of the fish life cycle.

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Tables

Table 1. Primer sequences and ZFID used in the present study.

Gene	Forward primer (5'- 3')	Reverse primer (5'- 3')	ZFIN ID
igf1	GGCAAATCTCCACGATCTCTAC	CGGTTTCTCTTGTCTCTCTCAG	ZDB-GENE-010607-2
igf2a	GAGTCCCATCCATTCTGTTG	GTGGATTGGGGTTTGATGTG	ZDB-GENE-991111-3
mstnb	GGACTGGACTGCGATGAG	GATGGGTGTGGGGATACTTC	ZDB-GENE-990415-165
nr3c1	AGACCTTGGTCCCCTTCACT	CGCCTTTAATCATGGGAGAA	ZDB-GENE-050522-503
hsp70.1	TGTTCAGTTCTCTGCCGTTG	AAAGCACTGAGGGACGCTAA	ZDB-GENE-990415-91
fads2	CATCACGCTAAACCCAACA	GGGAGGACCAATGAAGAAGA	ZDB-GENE-011212-1
elovl5	TGGATGGGACCGAAATACAT	GTCTCCTCCACTGTGGGTGT	ZDB-GENE-040407-2
elovl2	CACTGGACGAAGTTGGTGAA	GTTGAGGACACACCACCAGA	ZDB-GENE-060421-5612
chia.1	ACTGGGCGGAGCCTCAGTGT	GGGCTTGGGTGGGAAACCCAG	ZDB-GENE-040426-1994
chia.2	GGTGCTCTGCCACCTTGCCTT	GGCATGGTTGATCATGGCGAAAGC	ZDB-GENE-040426-2014
chia.3	TCGACCCTTACCTTTGCACACACCT	ACACCATGATGGAGAACTGTGCCGA	ZDB-GENE-040426-2891
chia.4	TGGACACCTCCACACGCTGC	ATGCCCACTAATCCGCCCGC	ZDB-GENE-030131-9279
chia.5	CCACGGCTCACAGGACAACATCA	GTCCGCAGACGACAGGCGAA	ZDB-GENE-071004-113
chia.6	TCCACGGCTCATGGGAGAGTGTC	AGCGCCCTGATCTCGCCAGT	ZDB-GENE-030131-1140
rplp0	CTGAACATCTCGCCCTTCTC	TAGCCGATCTGCAGACACAC	ZDB-GENE-000629-1
rpl13	TCTGGAGGACTGTAAGAGGTATGC	AGACGCACAATCTTGAGAGCAG	ZDB-GENE-031007-1

Table 2. Survival, standard length (mm), dry weight (mg) and specific growth rate (SGR % increase in dry weight day^{-1}) of zebrafish larvae fed two different BSF diets (group A and group B) and a commercial feed (control). No significant differences among groups were detected ($P > 0.05$).

	Control			Group A			Group B		
	7 dps	14 dps	21 dps	7 dps	14 dps	21 dps	7 dps	14 dps	21 dps
Survival	-	-	44 ± 14	-	-	38 ± 20	-	-	30 ± 5
Standard length	3.2 ± 0.7	4.5 ± 0.7	6.6 ± 1.3	3.2 ± 0.3	4.6 ± 0.5	5.8 ± 0.8	3.5 ± 0.2	4.2 ± 0.3	5.5 ± 1.1
Dry weight	0.1 ± 0.0	1.2 ± 0.3	4.8 ± 2.3	0.1 ± 0.0	0.6 ± 0.0	2.4 ± 0.8	0.1 ± 0.0	1.2 ± 0.4	1.9 ± 0.7
SGR	-	-	28.4 ± 1.8	-	-	22.5 ± 2.6	-	-	19.8 ± 4.6

Table 3. Dry weight, and lipid (dry matter basis) percentage in the insect substrates, experimental diets and zebrafish larvae collected at 21 dps. * indicates statistically significant difference among experimental groups ($P < 0.05$). N=3.

	Insect substrate		Experimental diet			Zebrafish larvae		
	Group A	Group B	Control	Group A	Group B	Control	Group A	Group B
% dry weight	52.3±0.9	89.6±0.4*	97.6±0.2	98.6±0.3	92.4±0.6*	16.7±2.7	23.6±3.2	15.9±3.0*
% lipid dw	3.4±0.1	3±0.7	11±1.0	13±1.0	25±1.0*	9.5±1.8	7.3±3.2	12.7±3.8

Table 4. Fatty acid composition (as mg g^{-1} dw) of insect substrates, experimental diets and zebrafish larvae collected at 21c dps. For each matrix, means within rows bearing different letters are significantly different ($P < 0.05$).

	Insect substrates		Experimental diets			Zebrafish larvae		
	Group A	Group B	Control	Group A	Group B	Control	Group A	Group B
10:0	0.15±0.06	0.003±0.001	<DL	1.41±0.06^{a}	2.30±0.2^{b}	<DL	0.07±0.03^{a}	0.12±0.02^{a}
12:0	0.20±0.07	0.026±0.008	0.16±0.04^{a}	27±3^{b}	44±4^{c}	1.18±0.40^{a}	8.3±1.1^{b}	21±2^{c}
13:0	0.03±0.01	<DL	0.051±0.007^{a}	0.106±0.004^{a}	0.059±0.003^{a}	0.041±0.008^{a}	0.06±0.02^{a}	0.07±0.01^{a}
14:0	3.1±1.2	0.045±0.007*	4.9±0.8^{a}	8.2±0.9^{b}	15.1±0.7^{c}	2.9±1.1^{a}	5.8±2.7^{a}	12.2±0.8^{b}
15:0	0.57±0.30	0.012±0.002	0.74±0.08^{a}	0.62±0.05^{a}	0.19±0.02^{a}	0.62±0.14^{a}	0.61±0.19^{a}	0.78±0.05^{a}
16:0	21±2	0.83±0.19*	16.8±1.8^{a}	9.3±1.1^{b}	15.6±0.4^{a}	15.1±1.6^{a}	10.4±1.2^{b}	26±1^{c}
16:1n9	0.06±0.02	0.006±0.003	18.9±1.6^{a}	20±2^{a}	19.1±0.2^{a}	0.30±0.08^{a}	0.42±0.16^{a}	1.62±0.13^{a}
16:1n7	0.40±0.1	0.015±0.004	5.5±0.7^{a}	6.9±0.8^{a}	5.7±0.2^{a}	3.78±1.7^{a}	5.34±1.9^{a}	6.35±0.3^{a}
17:0	0.80±0.35	0.014±0.001	0.79±0.06^{a}	0.60±0.05^{a}	0.18±0.02^{a}	0.80±0.2^{a}	0.79±0.3^{a}	1.75±0.08^{a}
18:0	8.9±1.2	0.32±0.04*	5.0±0.5^{a}	2.0±0.2^{b}	2.4±0.2^{b}	7.1±0.7^{a}	6.2±0.8^{a}	9.5±0.5^{a}
18:1n9	2.9±1.0	0.90±0.2*	13.7±1.4^{a}	8.3±1.1^{b}	14.9±0.4^{a}	11.9±1.7^{a}	10.1±1.1^{b}	15.6±0.7^{b}
18:1n7	0.28±0.11	0.039±0.009	2.2±0.2^{a}	0.52±0.05^{b}	0.57±0.09^{a}	1.96±0.63^{a}	1.62±0.74^{a}	3.47±0.08^{a}
18:2n6	0.69±0.09	2.32±0.5*	11.8±1.5^{a}	4.0±0.3^{b}	7.8±0.5^{c}	8.5±0.9^{a}	2.2±1.0^{b}	5.6±0.24^{a}
18:3n6	<DL	<DL	0.19±0.02^{a}	<DL	<DL	0.20±0.06^{a}	0.18±0.08^{a}	0.50±0.07^{a}
18:3n3	<DL	0.48±0.11	2.5±0.3^{a}	0.34±0.02^{b}	0.95±0.06^{ab}	1.06±0.2^{a}	0.17±0.08^{a}	1.04±0.14^{a}
20:0	2.42±0.63	0.033±0.001*	0.37±0.03^{a}	0.37±0.04^{a}	0.10±0.02^{a}	0.26±0.1^{a}	0.20±0.1^{a}	0.06±0.02^{a}
20:1n9	0.65±0.22	0.027±0.003	1.49±0.09^{a}	0.05±0.02^{a}	0.44±0.06^{a}	0.87±0.4^{a}	0.31±0.24^{a}	0.22±0.01^{a}
20:2n6	<DL	<DL	0.29±0.02^{a}	<DL	<DL	0.28±0.1^{a}	0.12±0.06^{a}	0.23±0.03^{a}
20:3n3	<DL	<DL	0.21±0.01^{a}	<DL	<DL	0.12±0.04^{a}	0.19±0.30^{a}	<DL
20:3n6	<DL	<DL	0.20±0.01^{a}	<DL	<DL	0.47±0.17^{a}	0.75±0.43^{a}	0.11±0.03^{a}
20:4n6	<DL	<DL	1.20±0.06^{a}	0.13±0.02^{a}	<DL	1.85±0.83^{a}	1.97±0.9^{a}	4.31±0.3^{a}
20:5n3	<DL	<DL	11.8±1.0^{a}	0.14±0.01^{b}	<DL	5.30±0.8^{a}	0.50±0.32^{b}	0.58±0.03^{b}
21:0	0.17±0.07	<DL	0.08±0.01^{a}	<DL	<DL	0.14±0.07^{a}	0.11±0.07^{a}	0.22±0.06^{a}
22:0	2.68±0.94	0.027±0.004*	0.27±0.03^{a}	0.48±0.07^{a}	<DL	0.28±0.22^{a}	0.25±0.2^{a}	<DL
22:1n9	<DL	<DL	1.01±0.05^{a}	0.015±0.007^{a}	<DL	0.51±0.4^{a}	0.18±0.19^{a}	<DL
22:6n3	<DL	<DL	23±2^{a}	<DL	<DL	16.7±1.8^{a}	4.7±0.5^{b}	3.8±0.4^{b}
24:1n9	<DL	<DL	0.80±0.07^{a}	0.036±0.008^{a}	<DL	0.45±0.12^{a}	0.18±0.17^{a}	<DL

Figures

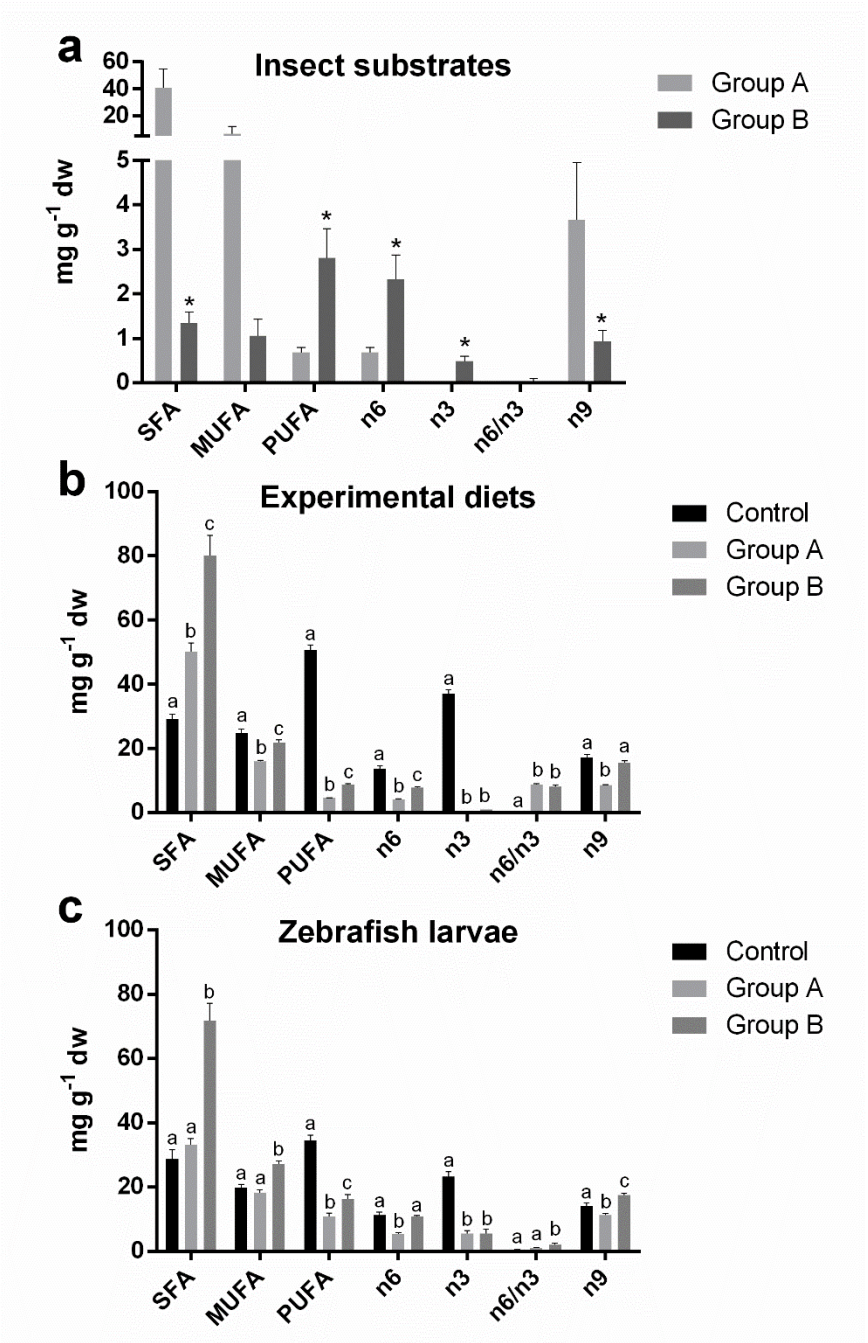

Fig. 1. Composition of Saturated (SFA), Mono-unsaturated (MUFA), and Poly-unsaturated (PUFA) fatty acids (as mg g^{-1} dw) in the insect substrates (a), experimental diets (b) and zebrafish larvae (c), and contribution of omega 3 (n3), omega 6 (n6), and omega 9 (n9) FAs to lipid profile. * and *different letters* indicate statistically significant differences among experimental groups ($P < 0.05$); N = 3.

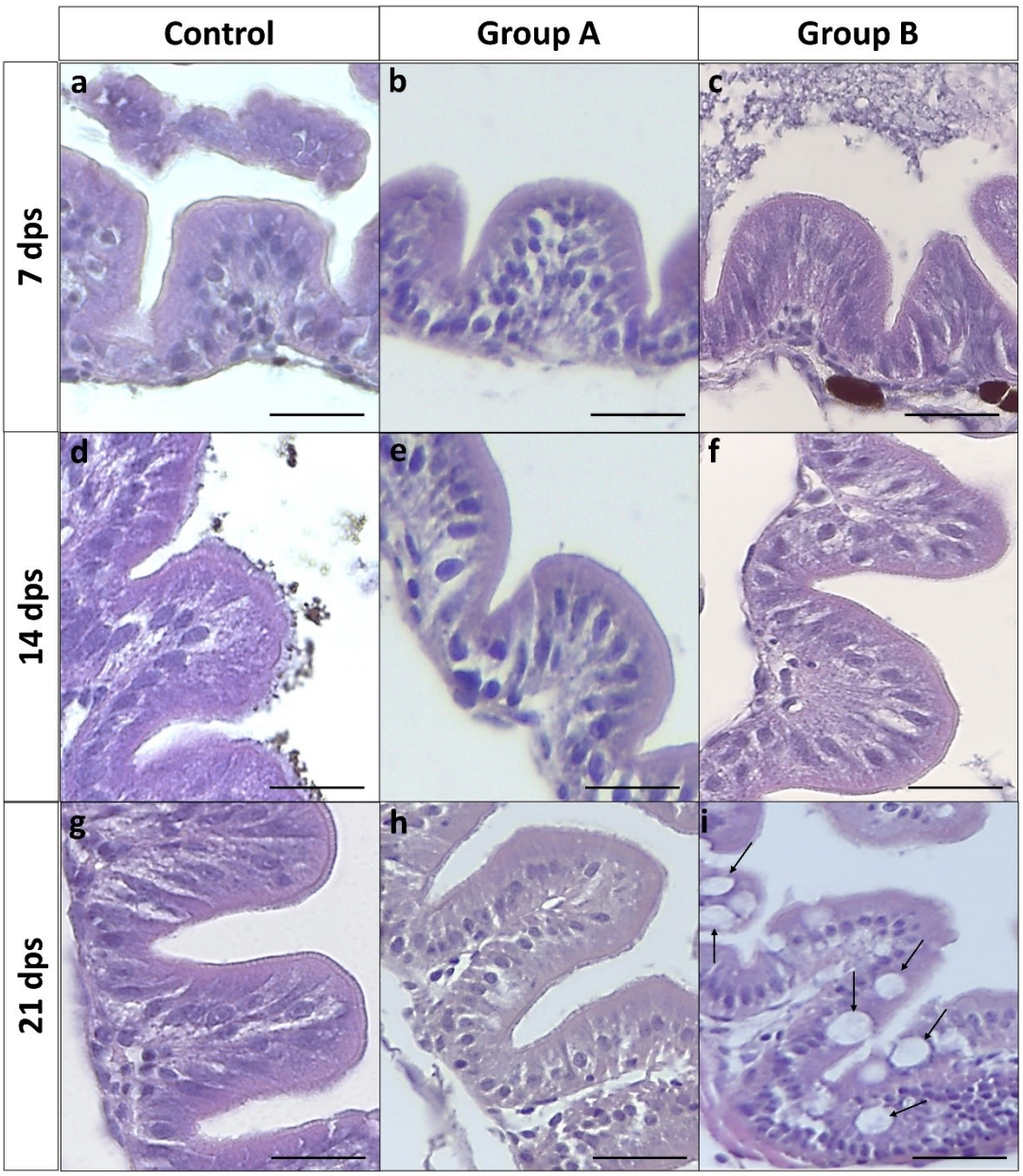

Fig. 2. Intestine morphology (E&E) of zebrafish larvae fed two different BSF diets (group A and group B) and a commercial feed (control) collected at 7, 14 and 21 dps (group A: b, e, h; group B: c, f, i; control: a, d, g). Group A: zebrafish larvae fed BSF grown on by-products obtained from roasting coffee process; group B: zebrafish larvae fed BSF grown on corn meal, fruit and vegetable mixture (50:50). A general increase in goblet cell number is evident at 21 dps in group B samples (i, Arrows). Scale bars: 25 µm.

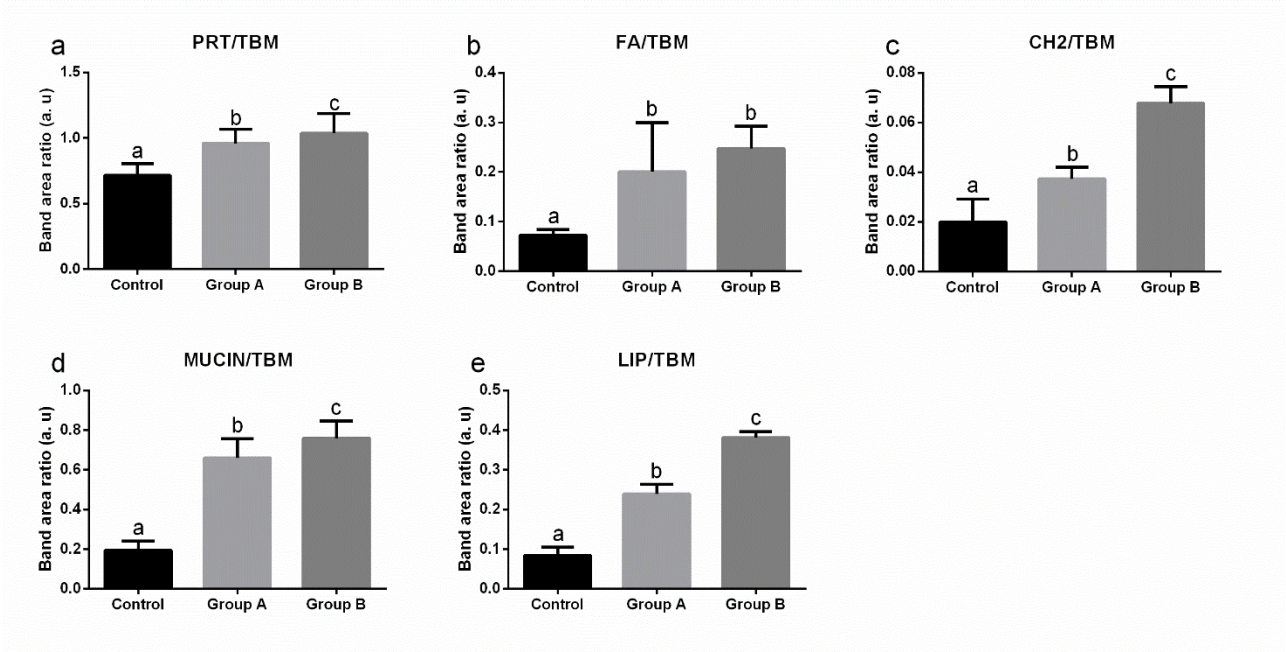

Fig. 3. Numerical variations of the following band area ratios calculated on IR spectra of intestine sections from 21 dps zebrafish larvae fed two different BSF diets (group A and group B) and a commercial feed (control group): LIP/TBM, FA/TBM, CH2/TBM, PRT/TBM, and MUCIN/TBM. Different letters indicate statistically significant differences among experimental groups ($P < 0.05$).

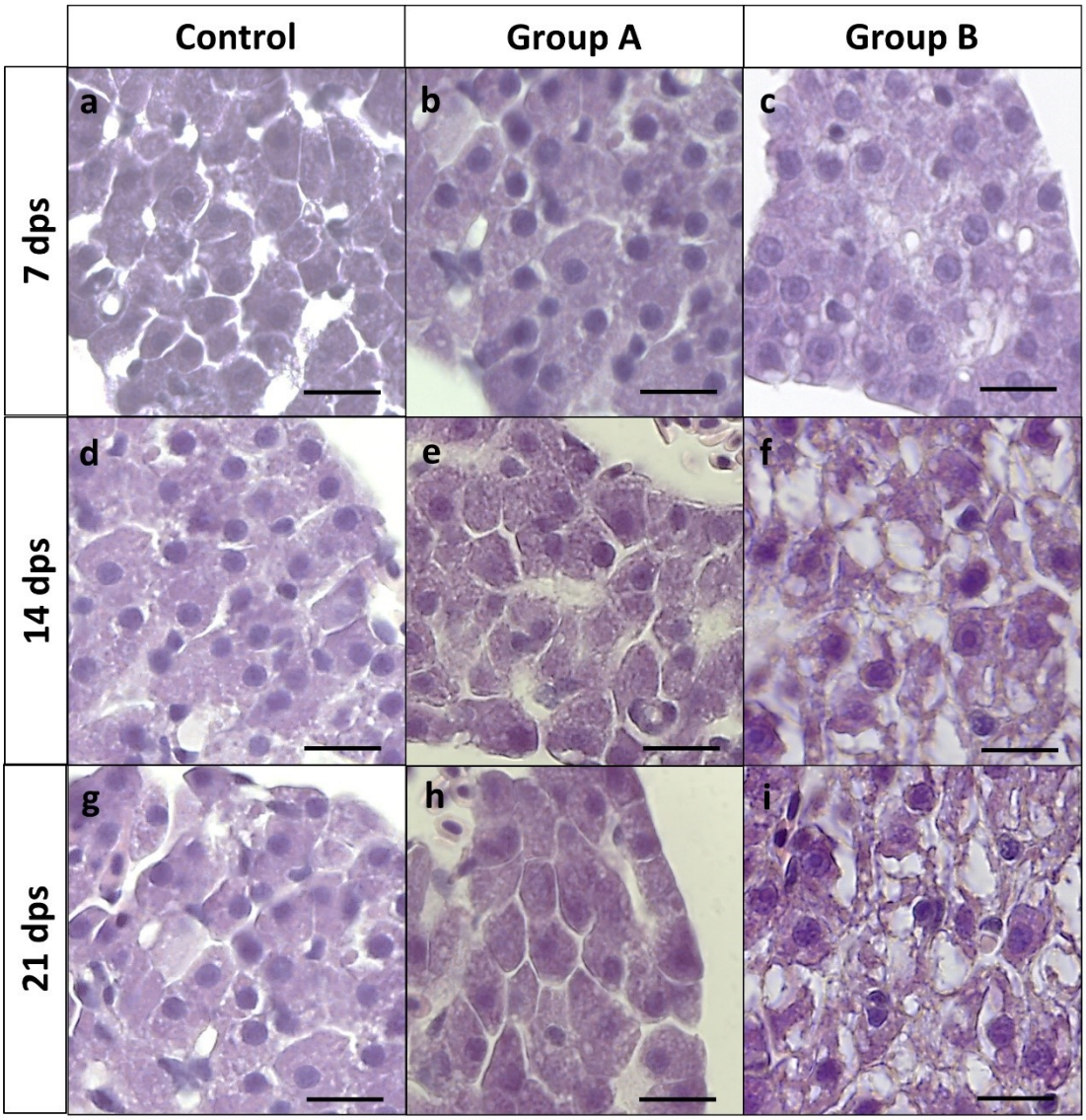

Fig. 4. Liver morphology (E&E) of zebrafish larvae fed two different BSF diets (group A and group B) and a commercial feed (control) collected at 7 , 14 and 21 dps. Group A: zebrafish larvae fed BSF grown on by-products obtained from roasting coffee process; group B: zebrafish larvae fed BSF grown on corn meal, fruit and vegetable mixture (50:50). At 7 dps, no histological alterations were observed in all experimental groups (a, b and c). No histological alterations were observed in (d and g) and in group A (a and h) at 14 and 21 dps. A general hepatic steatosis is evident at 14 and 21 dps in group B (f and i). Scale bars: 5 µm.

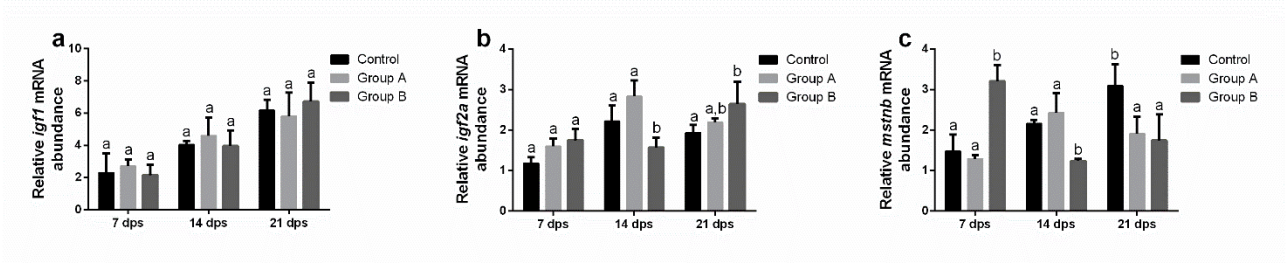

Fig. 5. Relative mRNA abundance of genes involved in fish growth (*igf1*, *igf2a* and *mstnb*) analyzed in zebrafish pools of 20 larvae, fed two different BSF diets (group A and B) and a commercial feed (control) collected at 7, 14 and 21 days post spawning (dps). Group A: zebrafish larvae fed BSF grown on by-products obtained from roasting coffee process; group B: zebrafish larvae fed BSF grown on corn meal, fruit and vegetable mixture (50:50). *Different letters* indicate statistically significant differences among experimental groups compared at the same experimental time ($P < 0.05$); N = 3.

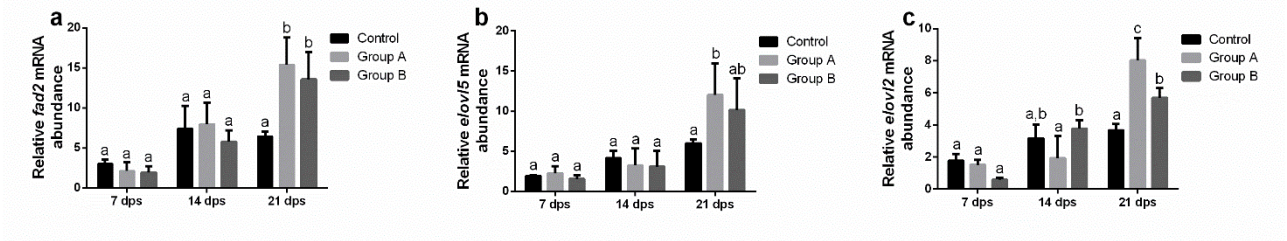

Fig. 6. Relative mRNA abundance of genes involved in long-chain polyunsaturated fatty acid biosynthesis (*fad2*, *elovl5* and *elovl2*) analyzed in zebrafish pools of 20 larvae, fed two different BSF diets (group A and B) and a commercial feed (control) collected at 7, 14 and 21 days post spawning (dps). Group A: zebrafish larvae fed BSF grown on by-products obtained from roasting coffee process; group B: zebrafish larvae fed BSF grown on corn meal, fruit and vegetable mixture (50:50). *Different letters* indicate statistically significant differences among experimental groups compared at the same experimental time ($P < 0.05$); N = 3.

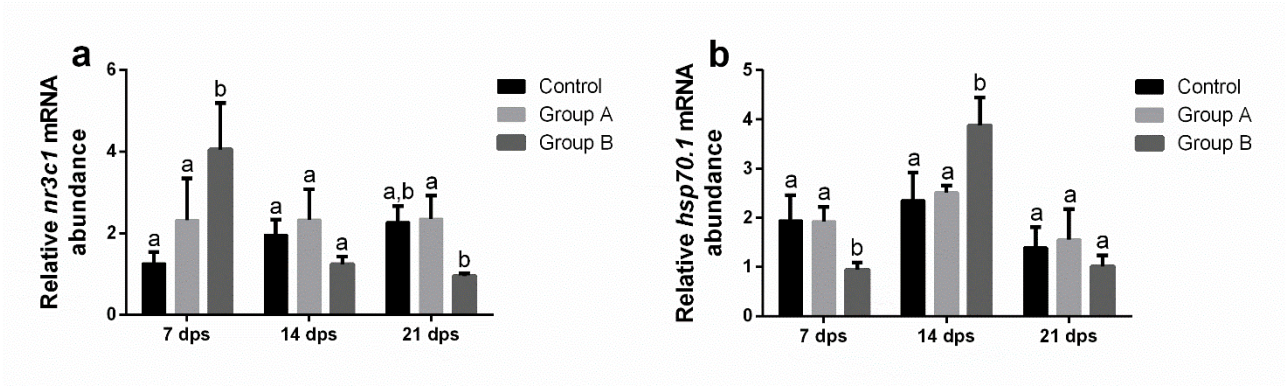

Fig. 7. Relative mRNA abundance of genes involved in stress response (*nr3c1* and *hsp70.1*) analyzed in zebrafish pools of 20 larvae, fed two different BSF diets (group A and B) and a commercial feed (control) collected at 7, 14 and 21 days post spawning (dps). Group A: zebrafish larvae fed BSF grown on by-products obtained from roasting coffee process; group B: zebrafish larvae fed BSF grown on corn meal, fruit and vegetable mixture (50:50). *Different letters* indicate statistically significant differences among experimental groups compared at the same experimental time ($P < 0.05$); N = 3.

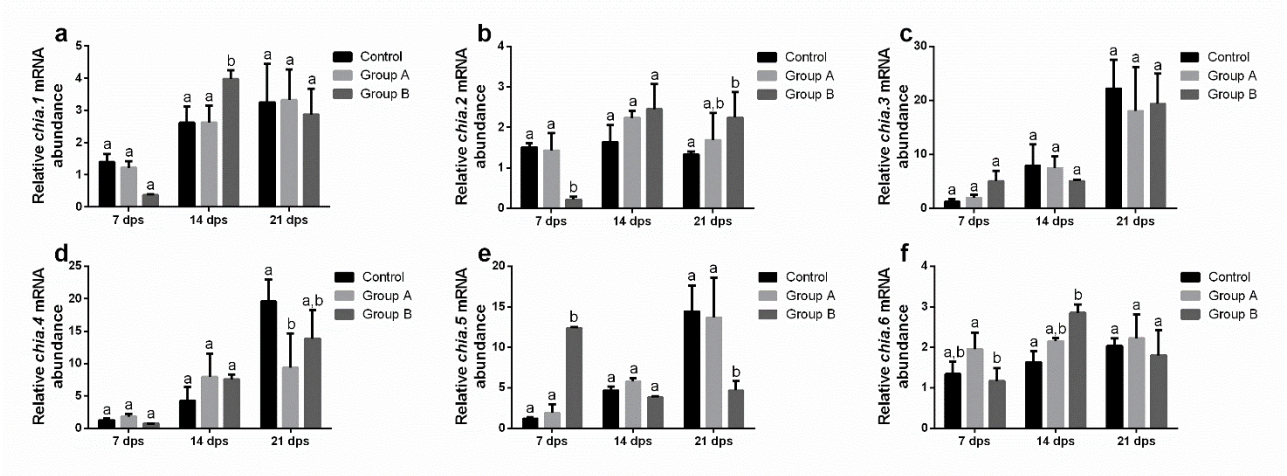

Fig. 8. Relative mRNA abundance of genes involved in enzymatic hydrolysis of chitin (*chia. 1, chia.2, chia.3, chia.4, chia.5* and *chia.6*) analyzed in zebrafish pools of 20 larvae, fed two different BSF diets (group A and B) and a commercial feed (control) collected at 7, 14 and 21 days post spawning (dps). Group A: zebrafish larvae fed BSF grown on by-products obtained from roasting

coffee process; group B: zebrafish larvae fed BSF grown on corn meal, fruit and vegetable mixture (50:50). *Different letters* indicate statistically significant differences among experimental groups compared at the same experimental time ($P < 0.05$); $N = 3$.

Chapter 2

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Partial dietary inclusion of *Hermetia illucens* (black soldier fly) full-fat prepupae in zebrafish feed: biometric, histological, biochemical and molecular implications.

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Abstract

Due to minimal environmental impact, compared to most conventional feed commodities, insects deserve a growing attention as candidate ingredients for aquafeeds. The present study tested, for the first time during zebrafish larval rearing, the effects of an increasing replacement (0-25-50%) of fish meal by Black Soldier Fly (BSF) full-fat prepupae meal. All diets were formulated to be isonitrogenous and isolipidic. A multidisciplinary approach, including biometrics, histology, gas chromatography mass spectrometry and molecular analyses was applied in order to better understand the biological responses of larval zebrafish to the different partial inclusions of BSF in the feed. Generally, results are promising, but a 50% of BSF meal inclusion in the diet affected both lipid composition and accumulation in the larvae.

Key words

Black soldier fly, gene expression, fatty acid profile, insect diet, aquaculture

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INTRODUCTION

Fish meal and fish oil represent the ideal ingredients for carnivorous fish diets due to their excellent nutritive properties, including high-protein content and adequate amino acid and fatty acid profile. Usually, high digestibility and palatability and the absence of anti-nutritional factors characterize this ingredient, ensuring optimal growth and health of cultured fish.[1]

Since fish meal and fish oil represent a crucial part of the current industrially produced diets,[2,3] the use of wild fish to feed farmed fish places direct pressures on fishery resources. In addition, fish meal and fish oil prices have dramatically risen in the past years[3] undermining the profitability of many aquaculture enterprises. Consequently, searching for alternatives to fish meal and oil represents a primary issue to be addressed in order to have a more sustainable aquaculture production.[4]

Over the last decades research has focused on testing different alternative ingredients to be used in aquafeed, including oilseeds, meat by-products, and microalgae. Most of the studies have focused on a possible application of vegetal proteins in fish feeds. Unfortunately, only a partial substitution is feasible because vegetal proteins may have unbalanced amino acid profile, poor protein digestibility and anti-nutritional substances content.[5] Furthermore, oilseed cultivation is often based on deforestation and high water consumption which led to additional environmental issues.[6]

Because of their adequate nutritional profile, their high content in pigments, antioxidants and bioactive compounds, microalgae have been investigated as an alternative feed ingredient[7]. However, despite their advantages, the use of microalgae faces severe barriers due to their current high costs and the difficulty in producing, concentrating and storing them.[7]

Scientists are now focusing on insects as a new protein source for animal feed.[8] Insects are an excellent source of nutrients,[9,10] they are highly productive and are able to

transform low-quality organic waste and manure into highly nutritious substances, playing a central role in the development of a circular bioeconomy.[10,11] Furthermore, the European Commission recently gave a boost to insect production allowing the use of processed insects in feed for aquaculture.[12] It is clear that partially substituting fish meal with insect meal will become a reality in animal farming in the EU.

Hermetia illucens (L.) (Diptera, Stratiomydae) (black soldier fly, BSF) is one of the most promising insect species because it shows an essential amino acid pattern similar to that of fish meal.[9] However, the fatty acid profile of insects does not always meet the nutritional requirements of fish since insects are normally rich in saturated fatty acids (SFA) and monounsaturated fatty acids (MUFA), rather than polyunsaturated ones. Another important consideration about the use of insects in aquafeed is the presence of chitin into their exoskeleton. Some fish species are capable to break down chitin glycosidic bond using endogenous enzymes (chitinases) or by the activity of chitinolytic bacteria.[13,14] However, the presence of chitin in the diet can reduce food intake, digestibility and nutrient absorption in cultured fish because of their limited digestive capacity for this polysaccharide.[15]

Studies have shown that fish feeds can include up to an intermediate amount of *H. illucens* without impairing growth performance nor feed digestibility.[16–19]

Zebrafish (*Danio rerio*) is one of the most studied experimental models in biomedical sciences, developmental biology, genetics, toxicology and aquaculture [20,21] due to its high reproductive rate and to the abundant information that has recently become available from genomic sequencing. To the best of our knowledge, there is no study performed on the larval development of zebrafish using partially substituited insect based diets. Improvements in larval diet efficiency and formulation is now a priority and the desirable goals are a reduction or even a complete substitution of unsustainable marine protein and

lipid feedstuff with more sustainable protein and lipid sources like those produced through insect rearing. A previous study by Vargas and collaborators (2018)[22] evidenced that a 100% BSF diet did not impair zebrafish larval development over a 21 days experiment. However, it is well established that over longer periods of time, insect meal could reduce fish growth and welfare because of a substantial high content in chitin[23] in addition to the deficiency in some essential amino acid.[22] For these reasons there is a strong need to test well balanced formulated diets with lower insect meal inclusions.

The present study investigates for the first time and through a multidisciplinary approach, including biometrics, histology, gas chromatography mass spectrometry and molecular analyses, the biological effects on zebrafish larval development of different partial inclusions of BSF meal in formulated isonitrogenous and isolipidic diets.

MATERIALS AND 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. 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 anesthetic (MS222; Sigma Aldrich, Milano, Italy).

Diets

Three experimental diets including increasing levels of BSF were formulated and produced at the facilities of the University of Udine. 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). Prepupae, once collected, were frozen (-80°C), freeze-dried and minced and through the use of liquid nitrogen.

The diets were formulated to be isonitrogenous (CP 40%) and isolipidic (EE 18%) with BSF full-fat prepupae replacing 25% (Group A) and 50% (Group B) of the fish meal of the basal diet (Control), respectively. Diets were analysed for proximate composition (AOAC, 1998) and gross energy content measured by an adiabatic bomb calorimeter (IKA C7000, Werke GmbH and Co., Staufen, Germany). The ingredient composition, proximate analysis and energy content of the experimental diets is shown in Table 1.

Fish

Zebrafish AB embryos were spawned and maintained 48h in a Tecniplast system (Varese, Italy), subjected to the following conditions: pH 7.0, NO_2 and NH_3 concentrations < 0.01 mg/L, NO_3 concentration < 10 mg/L, photoperiod 12L/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 according to the three test diets.

Experimental design

Zebrafish larvae were kept in nine 20 L tanks to set up the three experimental dietary treatments, each of 1500 fish (500 fish per tank). Fish were fed as follows: Control group, larvae fed the fish meal/oil based diet; Group A, larvae fed the diet including 25% of BSF full-fat prepupae meal; Group B, larvae fed the diet including 50% of BSF full-fat prepupae meal.

Zebrafish larvae were maintained in 20 L tanks fed the experimental diets (2% body weight, BW) twice a day.[24] The water in larval tanks (same chemical-physical characteristics of the parent's tank) was gently replaced ten times a day by a dripping system. The sides of the tank were covered with black panels to reduce light reflection).[25,26] Larvae were sampled at 7, 14 and 21 dps, euthanized with an excess of anesthetic (MS222 1g/L, Sigma Aldrich, Milano, Italy) and properly stored for further

analyses. All groups were fed (one feeding in the morning) on the rotifer *Brachionus plicatilis* (5 ind/mL) from 5 to 10 days post-spawning (dps) according to Lawrence et al. (2012).[27]

Biometry and survival

For biometric measurements, 10 larvae per tank (30 per group) were randomly collected from the different tanks, individually measured and bulk-weighed at each sampling time. The standard length was determined through the use of a sliding calliper (Measy 2000 Typ 5921, Swiss; precision: 0.1 mm) and the dry weight (calculated in pools of ten larvae) with an OHAUS Explorer (Greifensee, Switzerland) analytical balance (precision: 0.1 mg). Survival was determined by counting the remaining larvae in each tank at the end of the experiment (21 dps).

Lipid content and Fatty acid composition

The experimental diets and the larval fish samples collected at 21 dps (Control, Group A and Group B) were analyzed, in duplicate, for lipid content and fatty acid composition. Diets and fish larvae were minced, homogenized (homogenizer MZ 4110, DCG Eltronic, Monza, Italy) and freeze-dried (Edwards EF4, Crawley, Sussex, England) and lipid extraction was carried out on lyophilized powders following a microwave-assisted extraction (MAE).[28,29] Fatty acid methyl esters (FAMEs) were prepared according to Truzzi et al. (2017),[29] using the methyl ester of nonadecanoic acid (19:0; Dr. Ehrenstorfer GmbH, Augsburg, Germany) as internal standard. FAMEs were determined by an Agilent-6890 GC System (Milano, Italy) coupled to an Agilent-5973N 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 FAMEs. Instrumental conditions for the study matrices were set up, according to Truzzi et al. (2017).[29] The mass fraction of fatty acids, expressed as mg/g tissue dry weight, was

measured against the internal standard. For each sample, at least three runs were performed on the GC-MS. The precision of the proposed method was evaluated for the studied matrices as in Truzzi et al. (2014);[30] the intra-day and inter-day precision were, for major FAs, < 4% and < 7%, respectively, indicating a good repeatability of the analyses (data not shown). For FAs with a percentage <1% *vs* total FAs, intra-day and inter-day precision ranged from 5% to 20%, and from 8 to 25%, respectively. The estimated limits of detection (LOD) and limits of quantification (LOQ), calculated as in Truzzi et al. (2014),[31] ranged for each FAME from ~4 μg mL^{-1} to ~22 μg mL^{-1}, and from ~13 μg mL^{-1} to ~66 μg mL^{-1}, respectively.[29]

Histology

Five zebrafish larvae, in triplicate, randomly collected at each sampling time from the tanks of the three dietary treatments were fixed by immersion in Bouin's solution (Sigma-Aldrich, Milano, Italy) and stored at 4°C for 24h. Larvae were washed three times with ethanol (70%) for ten minutes and preserved in the same ethanol solution. Larvae were then dehydrated in crescent ethanol solutions (80, 95 and 100%), washed with the clearing agent "Histo-Clear" (Bio-Clear, 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 (Sigma-Aldrich, Milano, Italy). Sections were observed using a Leica MD750 (Nussloch, Germany) optical microscope connected with a Leica ICC50 HD (Nussloch, Germany) camera.

RNA extraction and cDNA synthesis

Total RNA extraction from 20 zebrafish larvae (in triplicate), randomly collected from the different tanks at each sampling time, was optimized using RNAzol® RT reagent (SIGMA-ALDRICH®, R4533) following the manufacturer's instructions. Total RNA

extracted was eluted in 20 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, 3 µg of RNA were used for cDNA synthesis, employing the High Capacity cDNA Reverse Transcription Kit (Bio-Rad) following the manufacturer's instructions.

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 set on a 96-well plate by mixing for each sample 1 µL cDNA diluted 1:20, 5 µL of 2x concentrated iQ™ Syber Green as the fluorescent intercalating agent, 0.3 µM forward primer, and 0.3 µM 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 60°C and 20 s at 72°C. Fluorescence was monitored at the end of each cycle. Dissociation curve analysis showed a single pick in all cases.

Relative quantification of the expression of genes involved in fish growth (*igf1*, *igf2a* and *mstnb*), stress response (*hsp70.1* and *nr3c1*), enzymatic hydrolysis of chitin (*chia.1*, *chia.2*, *chia.3*, *chia.4*, *chia.5* and *chia.6*), long-chain polyunsaturated fatty acid biosynthesis (*elovl2*, *elovl5* and *fads2*) and immune response (*tnfa* and *il6*) was performed using *arp* and *rpl13* as housekeeping genes to standardize the results (Table 2). Data were analyzed using the iQ5 optical system software version 2.0, including Genex Macro iQ5 Conversion and Genex Macro iQ5 files.

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 analyzed 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.

Statistical analysis

Survival data were analyzed by one-way ANOVA, with diet as the explanatory variable. The content and the fatty acid composition of diets and zebrafish larvae were analyzed by both t-test and one-way ANOVA. Standard length, dry weight and real-time PCR data were analyzed by two-way ANOVA, with both diet and dps as the explanatory variables. All ANOVA tests were followed by Tukey's post-hoc test. The statistical software package Prism5 (GraphPad Software) was used. Significance was set at $p < 0.05$ and all results are presented as mean ± SD.

RESULTS

Biometry and survival

Considering survival, no significant differences ($p>0.05$) were observed among the experimental groups. Group A reached the highest survival value (74±13%), while Control and Group B showed a 62±15% and 60±5% survival, respectively (Fig. 1).

Considering the standard length (Fig. 2a), no significant differences ($p>0.05$) were detected among the experimental groups at 7 and 14 dps. At the end of the experiment (21 dps), Control showed a significant lower value ($p<0.05$) in standard length respect to Group B (3.8±0.2 and 5.4±0.4 mm, respectively), while no significant differences were observed among Group A (4.9±0.3 mm) and the other groups. Finally, considering dry weight (Fig. 2b), no significant differences ($p>0.05$) were evident among the experimental groups at each sampling time, except for Group B that, at 21 dps, presented the highest value (3.8±1.0 mg).

Fatty acid content and composition

Diets. Table 3 reports the FAs composition (% FAMEs) of the experimental diets. The most relevant SFA 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 dietary treatments. Control diet showed the highest amount of DHA (22:6n3; 134.2 mg/g) and EPA (20:5n3; 68.5 mg/g) which significantly decreased in diet A (42.4 and 82.4 mg/g, respectively), and B (31.2 and 59.7 mg/g, respectively).

The effect of the dietary inclusion of the BSF meal on selected groups of fatty acids of the experimental diets is presented in Figure 3. Considering MUFA content, no significant differences ($p>0.05$) were detected among the tested diets. Differently, the increasing inclusion levels of BSF full-fat prepupae meal resulted in a significant increase ($p<0.05$) of dietary SFA content (337.6, 421.3 and 482.4 mg/kg for diet Control, A and B, respectively) and a parallel decrease in PUFA (polyunsaturated fatty acids) content (330.0, 230.0 and 185.0 mg/kg for diet Control, A and B, respectively; $p<0.05$). Regarding PUFA composition, a significant reduction ($p<0.05$) in n3 PUFA content was observed with the increasing dietary inclusion of BSF meal, while no significant differences ($p>0.05$) were detected among the tested diets regarding both n6 and n9 fatty acids. Finally, as concerns n6/n3 ratio, no significant differences ($p>0.05$) were observed between Control (0.50±0.11) and Group A (0.71±0.12) diets, while in Group B (0.85±0.12) diet the ratio was significantly ($p<0.05$) higher respect to Control.

Zebrafish larvae. Table 4 reported the fatty acid composition of zebrafish larvae. Considering SFA composition in the three experimental groups, the predominant fatty acid was the palmitic acid (16:0), followed by stearic acid (18:0). Both these FAs did not present significant differences ($p>0.05$) among the larvae. Group B fish showed a

significant ($p<0.05$) higher content of lauric (12:0) and myristic (14:0) acids with respect to Group A and Control fish. The most represented MUFA was oleic acid (18:1n9) and its content did not significantly ($p>0.05$) vary among the experimental zebrafish larvae. Finally, regarding PUFA, Control showed a high content of linoleic acid (18:2n6), followed by DHA (22:6n3) and EPA (20:5n3). A similar trend was observed in Group A and Group B larvae. Linoleic acid content did not present significant differences among the experimental groups whilst Group A fish were characterized by significant ($p<0.05$) lower EPA content respect to Control fish, while no significant differences were detected between these groups in terms of DHA content. Differently, both DHA and EPA contents of Group B fish were significantly ($p<0.05$) lower than those of Control fish.

Figure 4 reports the FAs composition (as mg/g dry weight) of zebrafish larvae collected at 21 dps. As concerns SFA content, no significant differences ($p>0.05$) were observed between Group A (40.8±1.7 mg/g) and Control (43.0±4.5 mg/g), while Group B showed a significant ($p<0.05$) higher content compared to the other groups (62.6±2.2 mg/g). Considering MUFA content, no significant differences ($p>0.05$) were detected between Control (40.8±3.5 mg/g) and Group B (45.0±2.0 mg/g), while the content of Group A (30.0±1.3 mg/g) was significantly ($p<0.05$) lower than the other experimental groups. Differently, both Group A (32.0±1.5 mg/g) and Group B (32.1±1.4 mg/g) presented a significant lower ($p<0.05$) PUFA content than Control (37.8±2.4 mg/g). Regarding n3 content, no significant differences ($p>0.05$) were detected between Group A (20.0±1.4 mg/g) and Control (21.4±1.9 mg/g), while the content of Group B (14.9±1.1 mg/g) was significantly ($p<0.05$) lower than that of the Control. In particular, as showed in Table 4, no significant differences ($p>0.05$) were detected between Control and Group A considering DHA and EPA contents whilst they were significantly ($p<0.05$) lower in Group B (DHA: 13.0±0.9 mg/g; EPA: 1.4±0.1 mg/g) compared to Control (DHA:

16.7±1.5 mg/g; EPA: 3.9±0.3 mg/g). No significant differences ($p>0.05$) were evident between Control and Group B in n6 (Control: 16.4±1.4 mg/g, Group B: 17.2±0.8 mg/g) and n9 (Control: 34.6±3.5 mg/g, Group B: 38.1±1.9 mg/g) contents. Conversely, Group A was significantly ($p<0.05$) lower than Control considering both n6 (12.0±0.5 mg/g) and n9 (25.9±1.3 mg/g) contents. Finally, regarding n6/n3, Control ratio (0.8±0.1) was significantly ($p<0.05$) higher than Group A (0.6±0.1), but significantly ($p<0.05$) lower respect to Group B (1±0.1).

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. No inflammatory events were observed in the intestine of all the analyzed samples at each sampling time (Fig. 5,A). Indeed, the morphology of mucosa appeared unaltered and characterized by the absence of appreciable inflammatory influx in all groups of zebrafish. On the contrary, differences in liver lipid accumulation were observed among the experimental groups (Fig. 5,B). At 7 dps, a consistent lipid accumulation was evident in Group B liver, but not in those of Control and Group A. Lipid vacuoles were still present in Group B livers at 14 dps, but not in those of Group A. At this sampling time, also Control showed the incidence of lipid accumulation. At 21 dps the presence of lipid vacuoles in the liver parenchima was comparable in Group A and Control, while Group B showed the highest accumulation (Fig. 5,B g,h,i).

Real-time PCR results

Real-time PCR analyses were performed on genes involved in fish growth (*igf1*, *igf2a* and *mstnb*), stress response (*hsp70* and *nr3c1*), enzymatic hydrolysis of chitin (*chia.1*,

chia.2, *chia.3*, *chia.4*, *chia.5* and *chia.6*), long-chain polyunsaturated fatty acids biosynthesis (*elovl2, elovl5* and *fads2*) and immune response (*tnfα* and *il6*).

Growth factors. As reported in Fig. 6a and Fig. 6b, *igf1* and *igf2a* gene expression evidenced similar patterns during the experimental period. For both *igf*s, no significant differences ($p>0.05$) were observed between Group A and Control at each sampling time, with the exception of *igf1* gene expression at 14 dps that was significantly ($p<0.05$) higher in Group A respect to Control. Conversely, Group B was significantly ($p<0.05$) higher than Control at each sampling time, except considering *igf2a* gene expression at 7 dps. A similar pattern was observed for *mstnb* gene expression (Fig. 6c); at 7 dps, Group A showed significantly ($p<0.05$) lower values than Control, while no significant differences ($p>0.05$) were evident between these groups at 14 and 21 dps. On the contrary, Group B showed a significantly ($p<0.05$) higher *mstnb* gene expression respect to Control at 14 and 21 dps.

Stress response. Considering *hsp70.1* gene expression (Fig. 7a), no significant differences ($p>0.05$) were observed among the experimental groups at 7 dps. Both Group A and Group B showed significantly ($p<0.05$) higher levels than Control at 14 dps, while at 21 dps, an opposite trend was observed. Regarding *nr3c1* gene expression (Fig. 7b), no significant differences ($p>0.05$) were detected among the experimental groups at 14 and 21 dps, while at 7 dps, both Group A and Group B exhibited a significantly ($p<0.05$) lower *nr3c1* gene expression compared to Control.

Chitinases. Regarding *chia.1* gene expression (Fig. 8a), no significant differences ($p>0.05$) were detected among experimental groups at 14 dps. Group A did not show significant ($p>0.05$) differences compared to Control at 7 dps, but was significantly ($p<0.05$) lower at 21 dps. Conversely, Group B showed a significantly ($p<0.05$) higher *chia.1* gene expression respect to Control and Group A both at 7 and 21 dps.

As concerns *chia.2* gene expression (Fig. 8b), no significant differences ($p>0.05$) were observed among Control and the treated groups at 14 dps. At 7 dps, no significant differences ($p>0.05$) were evident between Group A and Group B, while Group B resulted significantly ($p<0.05$) higher than Control. At 21 dps, Group A was characterized by a significantly ($p<0.05$) lower *chia.2* gene expression than Control, while Group B showed an opposite trend.

Expression of *chia.3* (Fig. 8c) at 7 dps did not show significant differences ($p>0.05$) among the experimental groups. At 14 dps no significant differences ($p>0.05$) were observed between Group A and Control, while Group B presented a significantly ($p<0.05$) lower *chia.3* gene expression than Control. At 21 dps, instead, only Group A was significantly lower ($p<0.05$) than Control.

With regard to *chia.4* gene expression (Fig. 8d), no significant differences ($p>0.05$) were detected among the experimental groups at 7 and 14 dps. Both Group A and Group B reached a significantly ($p<0.05$) higher gene expression than Control at 21 dps. Finally, both *chia.5* and *chia.6* gene expression (Fig. 8e,d), at 7 dps, were significantly ($p<0.05$) higher in Group A with respect to Control, while no significant differences ($p>0.05$) were detected between Control and Group B. At 14 dps, significant differences ($p<0.05$) were evident among the experimental groups, with Control higher than Group A, but lower than Group B. At 21 dps, Group A and Group B were characterized by a significantly ($p<0.05$) lower *chia.5* gene expression with respect to Control, while no significant ($p>0.05$) differences were observed among the experimental groups considering *chia.6* gene expression.

Lipid metabolism. Regarding *elovl2* gene expression (Fig. 9a), Group A and Control did not show significant differences ($p>0.05$) at all sampling times. Conversely, Group B was characterized by a significantly ($p<0.05$) higher *elovl2* gene expression than Control and

Group A at 7 and 21 dps. No significant differences ($p>0.05$) were observed among the treated groups and Control at each sampling times considering *elovl5* gene expression (Fig.9b). As showed in Figure 9c, *fads2* gene expression was not characterized by significant differences ($p>0.05$) among the experimental groups at 7 and 14 dps. Finally, at 21 dps, Group A showed significantly ($p<0.05$) lower values than Control, which did not show significant differences ($p>0.05$) compared to Group B.

Immune response. Regarding *tnfa* gene expression (Fig. 10a), Group A did not show significant differences ($p>0.05$) with respect to Control at all sampling times. Conversely, Group B was significantly ($p<0.05$) higher than Control and Group A at 7 and 21 dps. As concerns *il6* gene expression (Fig. 10b), no significant differences ($p>0.05$) were detected among the experimental groups at 14 dps. At 7 dps, both Group A and Group B were significantly ($p<0.05$) higher than Control, while, at 21 dps, only Group B was characterized by a significantly ($p<0.05$) higher *il6* gene expression with respect to Control.

DISCUSSION

The desirable goal of the aquaculture industry is to replace fish meal and fish oil with more sustainable ingredients.[32] However, alternatives must ensure economic feasibility and a proper fish growth and health by providing adequate levels of nutrients.[4] Insects can exemplify good candidates for a more sustainable aquaculture, representing a valid alternative to fish meal and fish oil. However, their application in aquafeeds still needs to be explored.[11] Many studies have been carried out in the last years on the inclusion of insects in aquafeed formulation; however, results are still controversial and further research is necessary. For example, replacing up to 25 or 50% of fish meal with mealworm, *Tenebrio molitor* (Coleoptera, Tenebrionidae) had no adverse effects on

weight gain in *Sparus aurata*, but fish fed diet with 50% insect substitution showed a growth reduction and less feed conversion ratio compared to control.[33] Similar results were obtained in African catfish (*Clarias gariepinus*), with a replacement of 25% of fish meal by variegated grasshopper, *Zonocerus variegatus* (Orthoptera, Pyrgomorphidae), that improved both growth rate and nutrient utilization. Conversely, when the inclusion of *Z. variegatus* was increased above 50%, growth of *C. gariepinus* was negatively influenced.[34]

One of the limiting factors of including insects in aquafeeds is their fatty acid profile that does not always match the nutritional requirements of fish. It is known that in insects, the quantity and quality of fatty acids are species specific, vary with the developmental stage and can be modified by the growth substrate.[35,36] Among several insect species, BSF is one of the most promising because it shows an essential amino-acid profile similar to that of fish meal.[9] In addition, its lipid profile can be manipulated by changing the composition of the growth substrate.[35] The present study investigated for the first time the formulation of new test diets including different substitution levels of fish meal and fish oil with full-fat BSF meal, during zebrafish larviculture. Zebrafish is a widely used model organism but its dietary preferences and its nutritional requirements are mostly unknown.[20] Results obtained from the present study may be used to generalize how several biological processes occur in related species, especially in farmed fish, improving our understanding on the mechanisms involved in fish nutrition and growth.[37]

In order to gain a deeper knowledge about the effects of the inclusion of BSF meal in aquafeeds a comprehensive multidisciplinary approach was applied.

Generally, all the tested experimental diets did not show significant negative effects on larval survival. Indeed, the experimental groups showed comparable survival rates, in accordance with previous studies on various fish species.[18,19,38,39] Growth (standard length

and mean dry weight) was also similar among the groups up to 14 dps; however, even if the three diets were isoenergetic, at 21 dps, Group B larvae were significantly bigger (standard length and mean dry weight) respect to both Control and Group A larvae. All biometric data were fully supported by the molecular results about growth factors (*igfs* and *mstn*) and were in accord with previous studies on other fish species such as rainbow trout (*Oncorhynchus mykiss*), channel catfish (*Ictalurus punctatus*) and blue tilapia (*Oreochromis aureus*).[40,41]

One of the main problems of the use of insect meal in aquafeed is related to its FA profile which in turn affects the fish quality as reported by St-Hilaire et al. (2007).[35] This could be problematic for consumers who desire fish with high concentrations of long-chain unsaturated fatty acids.

In the present study, the three different diets showed a similar total lipid content but a different FA composition. In particular, diets with higher inclusions of BSF meal showed an increase in SFA and a decrease in PUFA.

However, regarding PUFAs, a reduction in these severe differences was observed in zebrafish larvae fed the different diets. A reasonable explanation of this result is the ability of many freshwater species, including zebrafish, to convert shorter-chain precursors in highly unsaturated FAs through the activation of specific elongase and desaturase (*elovl2*, *elovl5* and *fad*).[42]

As regards SFA, differences between diets and larval composition were less evident. In fact, a significant increase in SFA larval content was observed only with the highest BSF meal inclusion (Group B). Group B larvae where particularly rich in lauric acid (C12). This result is in line with other studies[22,43] reporting that lauric acid, a medium chain fatty acid particularly abundant in insects (mainly in *H.illucens*), is usually preferentially

utilized as energy source and efficiently absorbed, digested and β-oxidised if provided in adequate amounts through the diet.[44]

Even if the three offered diets were isolipidic, larval fish hepatic accumulation was very different. In fact, only group B larvae showed a severe lipid steatosis in the liver.

Since n-3 PUFA are known to limit triglyceride deposition in the liver, steatosis has been reported to be related to a PUFA deficiency, and to high n-6/n-3 ratio.[45,46] In addition, a recent review reports that an increase in the dietary SFA may play an important role in the development of hepatic steatosis,[47] causing liver dysfunction by promoting endoplasmic reticulum stress and apoptosis.[48–50]

As a consequence, the observed lipid steatosis of Group B larvae can be related to the quality rather than the quantity of the dietary fat provided through the three different diets. In fact, the above mentioned *scenario* was fully represented only in Group B: lower n-3 and higher SFA content and a higher n6/n3 ratio respect to Control and Group A.

Medium-chain fatty acids have also been reported to possess positive properties such as antibacterial and antiviral activity and have demonstrated positive effects improving gut health under inflammatory conditions.[51–53] Insects are rich in chitin which may induce intestinal inflammation and a reduction in fish welfare and growth.[54,55] However, in the present study, no signs of intestinal inflammation were observed, possibly due to the high supplementation of medium-chain fatty acids through the diet. These data were also generally supported by molecular analysis on stress markers that did not show significant variations in their expression during the experiment.

However, it should be pointed out that the present study was only 21 days long and pathological signs could become evident over a longer period of time. As a consequence, molecular markers can be useful to precociously detect physiological responses. In the present study, genes involved in the immune response (*il6* and *tnfα*) were highly

expressed in Group B compared to Control and Group A. This result was not obvious and can possibly be related to an upcoming intestinal inflammation or to the already evident hepatic steatosis.

Finally, different fish species may have different chitinolytic activity. Some species, like turbot (*Scophthalmus maximus*), do not have any chitinolytic activity during the early life stages,[15] while others, like cobia (*Rachycentron canadum*), showed very high endochitinolytic activity.[56] Generally, the analysis of the six zebrafish chitinases in larvae fed different diets showed that chitinase gene expression was not totally dependent on the amount of chitin provided through the diet. This result was not obvious and a possible involvement of intestinal bacteria with chitinolytic activity should be considered in future studies.

CONCLUSION

The present study tested for the first time the partial dietary inclusion of *Hermetia illucens* meal in zebrafish larval diets and the physiological responses of the larvae. Generally, results are promising but a 50% BSF meal dietary inclusion affected both lipid composition and accumulation of the larvae. Further studies are needed to better understand the physiological responses of fish, including very low BSF-meal substitutions which may play a immunomodulatory role.

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Tables

	Control	Group A	Group B
Ingredients (g/kg)			
Fish meal, Chile, super prime	420	315	210
Peas, protein concentrate	55	78	100
Hermetia illucens	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 §	20	20	20
Binder	20	20	20
L-methionine	0	1	1
Proximate composition (%)			
Dry matter	4.2±0.03	5.5±0.18	5.3±0.42
Crude protein	40.0±0.47	40.2±0.39	41.1±0.10
Crude lipid	18.6±0.14	17.7±0.20	17.0±0.13
Ash	14.2±0.23	14.1±0.31	12.2±0.59
N-free extractive (NFE)	23.0±0.31	22.5±0.60	24.4±0.99
Gross Energy (MJ/kg)	22.1±0.11	22.3±0.03	21.3±0.06

Table 1. Ingredient composition, proximate analysis and gross energy content of the test diets. §Composition of mineral mix (g/kg diet): Ca HPO4 *2H2O, 27.5; K2HPO4, 19.0; NaCl, 6.1; MgO, 2.0; FeCO3, 1.75; KI, 0.15; ZnO, 0.11; MnO, 0.07; CuSO4, 0.02; sodium selenite, 0.002. Composition of vitamin mix (mg/kg diet): thiamin HCl, 40; riboflavin, 40; pyridoxine HCl, 40; cyanocobalamine, 0.2; niacin, 300; calcium panthothenate, 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.

Table 2. Primer sequences and ZFID used in the present study

Gene	*Forward primer (5'- 3')*	*Reverse primer (5'- 3')*	*ZFIN ID*
igf1	5'-GGCAAATCTCCACGATCTCTAC-3'	5'-CGGTTTCTCTTGTCTCTCTCAG-3'	ZDB-GENE-010607-2
igf2a	5'-GAGTCCCATCCATTCTGTTG-3'	5'-GTGGATTGGGGTTTGATGTG-3'	ZDB-GENE-991111-3
mstnb	5'-GGACTGGACTGCGATGAG-3'	5'-GATGGGTGTGGGGATACTTC-3'	ZDB-GENE-990415-165
hsp70.1	5'-TGTTCAGTTCTCTGCCGTTG-3'	5'-AAAGCACTGAGGGACGCTAA-3'	ZDB-GENE-990415-91
nr3c1	5'-AGACCTTGGTCCCCTTCACT-3'	5'-CGCCTTTAATCATGGGAGAA-3'	ZDB-GENE-050522-503
chia.1	5'-ACTGGGCGGAGCCTCAGTGT-3'	5'-GGGCTTGGGTGGGAAACCCAG-3'	ZDB-GENE-040426-1994
chia.2	5'-GGTGCTCTGCCACCTTGCCTT-3'	5'-GGCATGGTTGATCATGGCGAAAGC-3'	ZDB-GENE-040426-2014
chia.3	5'-TCGACCCTTACCTTTGCACACACCT-3'	5'-ACACCATGATGGAGAACTGTGCCGA-3'	ZDB-GENE-040426-2891
chia.4	5'-TGGACACCTCCACACGCTGC-3'	5'-ATGCCCACTAATCCGCCCGC-3'	ZDB-GENE-030131-9279
chia.5	5'-CCACGGCTCACAGGACAACATCA-3'	5'-GTCCGCAGACGACAGGCGAA-3'	ZDB-GENE-071004-113
chia.6	5'-TCCACGGCTCATGGGAGAGTGTC-3'	5'-AGCGCCCTGATCTCGCCAGT-3'	ZDB-GENE-030131-1140
elovl2	5'-CACTGGACGAAGTTGGTGAA-3'	5'-GTTGAGGACACACCACCAGA-3'	ZDB-GENE-060421-5612
elovl5	5'-TGGATGGGACCGAAATACAT-3'	5'-GTCTCCTCCACTGTGGGTGT-3'	ZDB-GENE-040407-2
fads2	5'-CATCACGCTAAACCCAACA-3'	5'-GGGAGGACCAATGAAGAAGA-3'	ZDB-GENE-011212-1
tnfa	5'-TTGTGGTGGGGTTTGATG-3'	5'-TTGGGGCATTTTATTTTGTAAG-3'	ZDB-GENE-050317-1
il6	5'-CTGGAGGCCATAAACAGCCA-3'	5'-TGCGAGTCCATGCGGATTTA-3'	ZDB-GENE-120509-1
arpc1a	5'-CTGAACATCTCGCCCTTCTC-3'	5'-TAGCCGATCTGCAGACACAC-3'	ZDB-GENE-040116-1
rpl13	5'-TCTGGAGGACTGTAAGAGGTATGC-3'	5'-AGACGCACAATCTTGAGAGCAG-3'	ZDB-GENE-031007-1

Table 3. Fatty acid composition (% FAMEs) of experimental diets. Means within rows bearing different letters differ significantly ($p<0.05$).

	Control	**Group A**	**Group B**
10:0	n.d.	0.36±0.03 [a]	0.74±0.04 [a]
12:0	0.14±0.05 [a]	6.49±0.68 [b]	13.05±1.37 [c]
13:0	0.05±0.02 [a]	0.04±0.05 [a]	0.07±0.04 [a]
14:0	4.33±0.16 [a]	4.02±0.31 [a]	5.68±1.05 [b]
15:0	0.70±0.09 [a]	0.43±0.08 [a]	0.54±0.26 [a]
16:0	15.07±1.63 [b]	24.11±2.81 [a]	21.62±1.21 [a]
16:1n9	0.18±0.04 [a]	0.13±0.05 [a]	0.18±0.02 [a]
16:1n7	5.30±0.36 [a]	3.88±0.91 [b]	5.25±0.84 [a]
17:0	0.77±0.11 [a]	0.48±0.04 [a]	0.59±0.13 [a]
18:0	4.89±0.74 [a]	5.44±1.11 [a]	5.08±0.23 [a]
18:1n9	13.24±0.55 [a]	27.85±1.65 [b]	24.97±1.76 [b]
18:1n7	2.14±0.38 [a]	0.99±0.16 [b]	0.98±0.31 [b]
18:2n6	11.24±1.53 [a]	8.95±1.46 [ab]	8.03±1.49 [b]
18:3n6	0.19±0.03 [a]	0.07±0.04 [ab]	0.05±0.06 [b]
18:3n3	2.44±0.13 [a]	1.13±0.23 [b]	0.95±0.35 [b]
20:0	0.36±0.07 [a]	0.42±0.14 [a]	0.45±0.07 [a]
20:1n9	1.45±0.35 [a]	0.86±0.22 [a]	0.94±0.20 [a]
20:2n6	0.28±0.01 [a]	0.10±0.0 [b]	0.08±0.03 [c]
20:3n6	0.20±0.04 [a]	0.07±0.08 [b]	0.05±0.03 [b]
21:0	0.07±0.05 [a]	0.03±0.03 [a]	0.02±0.02 [a]
20:4n6	1.21±0.14 [b]	0.49±0.13 [a]	0.41±0.06 [a]
20:3n3	0.22±0.07 [a]	0.10±0.14 [a]	0.07±0.04 [a]
20:5n3	11.34± 0.24 [a]	4.24±0.49 [b]	3.12±0.74 [b]
22:0	0.26±0.03 [a]	0.27±0.01 [a]	0.36±0.07 [a]
22:1n9	0.92±0.13 [a]	0.39±0.05 [b]	0.35±0.08 [b]
24:0	0.01±0.01 [a]	0.05±0.02 [a]	0.04±0.05 [a]
22:6n3	22.23±0.89 [c]	8.24±0.36 [b]	5.97±0.72 [a]
24:1n9	0.77±0.38 [a]	0.37±0.08 [a]	0.38±0.08 [a]

Table 4. Fatty acid composition (as mg/g dw) of zebrafish larvae collected at 21 dps. Means within rows bearing different letters are significantly different ($p < 0.05$).

	Control	**Group A**	**Group B**
10:0	n.d. [a]	0.12±0.03 [a]	0.17±0.13 [a]
12:0	0.29±0.04 [a]	3.63±0.97 [b]	12.31±2.06 [c]
13:0	0.07±0.01 [ab]	0.04±0.00 [a]	0.11±0.04 [b]
14:0	3.50±0.32 [a]	3.47±0.53 [a]	8.24±1.92 [b]
15:0	0.81±0.02 [ab]	0.60±0.02 [a]	1.10±0.32 [b]
16:0	24.47±1.31 [a]	20.20±2.66 [a]	26.26±5.81 [a]
16:1n9	0.89±0.21 [ab]	0.63±0.09 [a]	1.35±0.32 [b]
16:1n7	3.21±0.62 [ab]	2.08±0.55 [a]	3.94±0.84 [b]
17:0	0.95±0.03 [ab]	0.75±0.06 [a]	1.23±0.29 [b]
18:0	12.47±0.56 [a]	11.66±0.35 [a]	12.73±1.58 [a]
18:1n9	32.05±0.71 [a]	24.52±5.35 [a]	35.96±5.79 [a]
18:1n7	3.06±0.04 [b]	2.09±0.36 [a]	3.01±0.48 [b]
18:2n6	14.00±0.52 [a]	9.71±2.29 [a]	14.05±2.33 [a]
18:3n6	0.20±0.00 [b]	0.11±0.02 [a]	0.19±0.03 [b]
18:3n3	0.48±0.03 [b]	0.21±0.05 [a]	0.31±0.07 [a]
20:0	0.48±0.00 [b]	0.35±0.05 [a]	0.41±0.04 [ab]
20:1n9	1.38±0.21 [b]	0.56±0.09 [a]	0.61±0.10 [a]
20:2n6	0.88±0.08 [a]	0.65±0.17 [a]	0.83±0.13 [a]
20:3n6	0.88±0.01 [a]	0.98±0.16 [a]	1.54±0.17 [b]
20:4n6	0.45±0.01 [a]	0.54±0.02 [b]	0.60±0.05 [b]
20:3n3	0.37±0.02 [b]	0.20±0.05 [a]	0.23±0.03 [a]
20:5n3	3.86±0.32 [b]	1.78±0.28 [a]	1.40±0.14 [a]
22:1n9	0.25±0.00 [b]	0.15±0.02 [a]	0.15±0.01 [a]
22:6n3	16.73±1.47 [b]	17.86±1.63 [b]	12.97±0.85 [a]

Figures

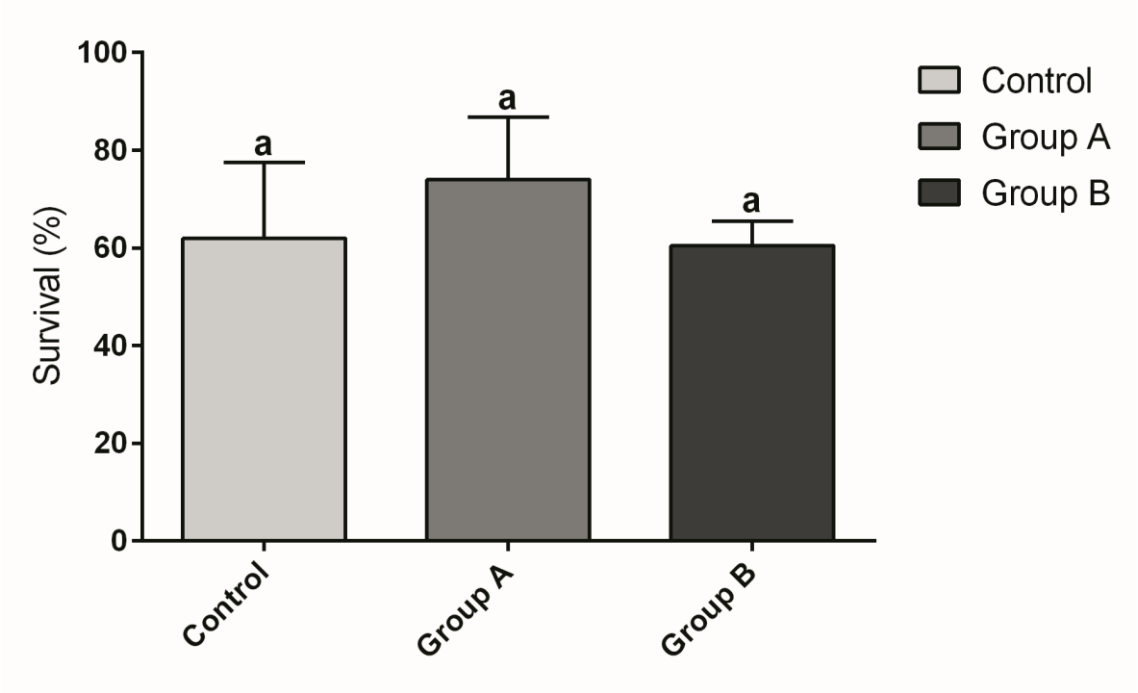

Fig. 1. Survival (in %) of zebrafish larvae fed diet based on fish meal (Control) and diets with 25% (Group A) or 50% (Group B) replacement of fish meal with BSF meal.

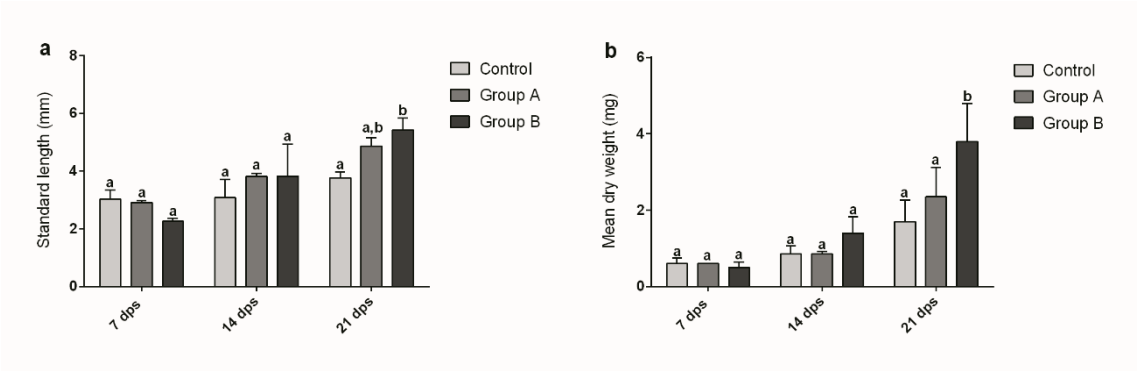

Fig. 2. (a) Standard length (mm) and (b) mean dry weight (mg) of zebrafish larvae fed diet based on fish meal (Control) and diets with 25% (Group A) or 50% (Group B) replacement of fish meal with BSF meal. Mean dry weight was referred to pools of 10 animals for each group. Different letters indicate statistically significant differences among experimental groups compared within the same sampling time ($p<0.05$). Values are presented as mean ± SD (n = 3).

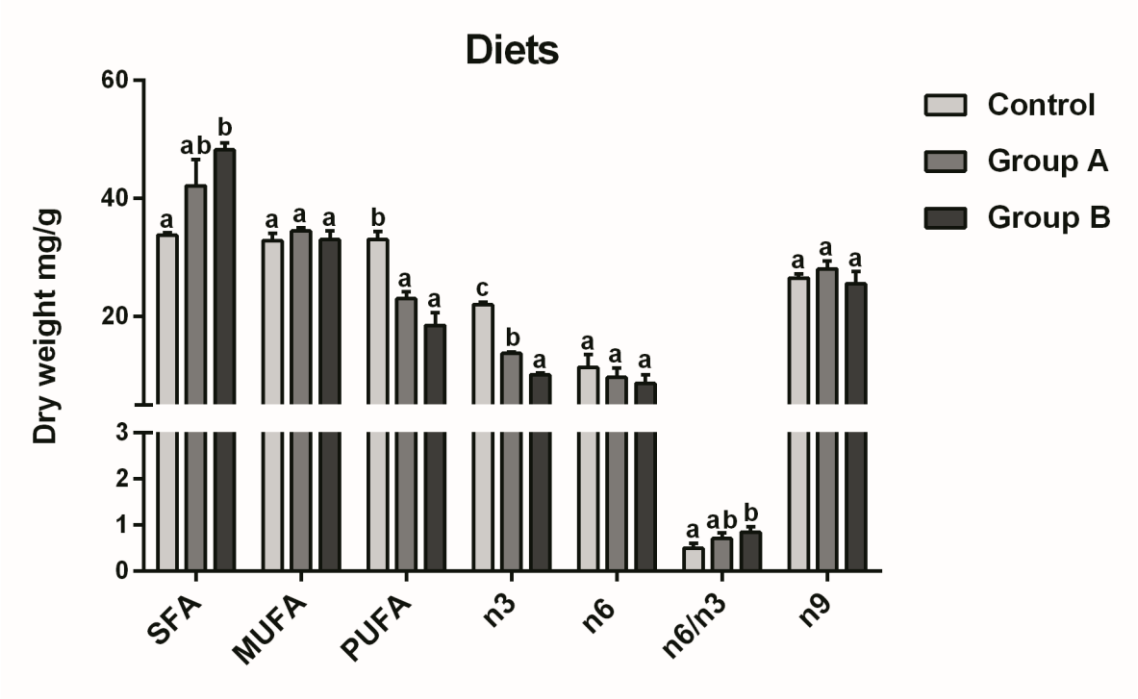

Fig. 3. Content of saturated (SFA), mono-unsaturated (MUFA) and poly-unsaturated (PUFA) fatty acids in the experimental diets, contribution of n3, n6 and n9 fatty acids to lipid profiles (as mg/g dry weight) and n6/n3 ratio. Different letters indicate statistically significant differences among experimental groups compared within the same fatty acid class ($p<0.05$). Values are presented as mean ± SD ($n = 3$). Control diet was based on fish meal, while Group A and Group B diets were characterized by 25 or 50% replacement of fish meal with BSF meal, respectively.

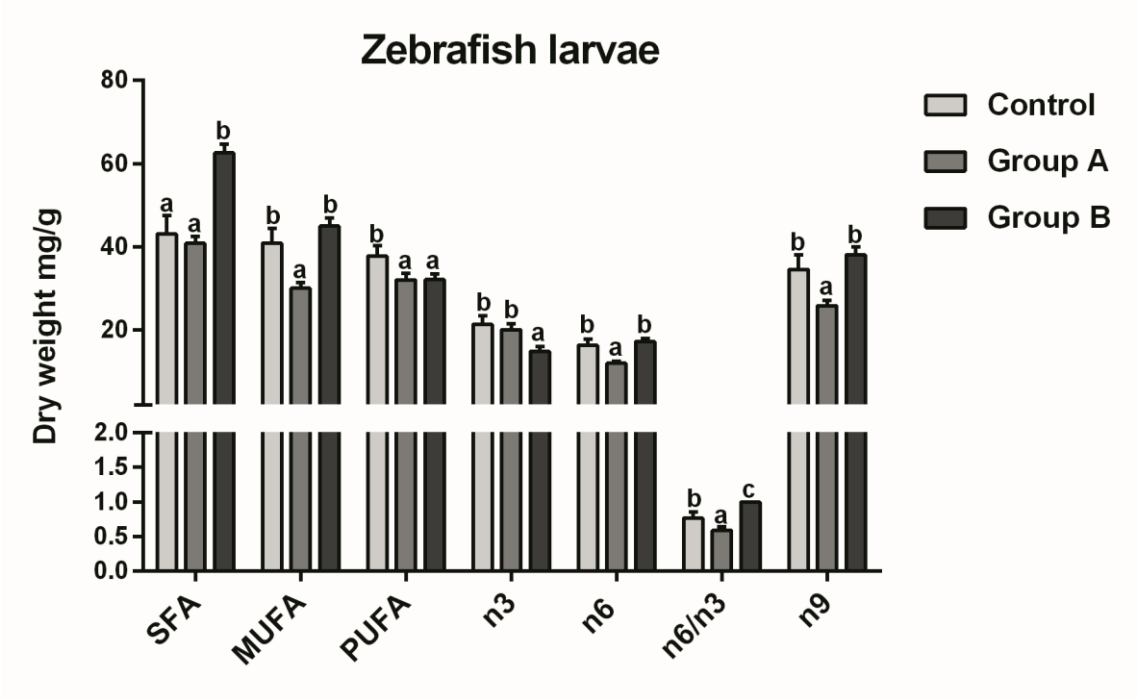

Fig. 4. Content of saturated (SFA), mono-unsaturated (MUFA) and poly-unsaturated (PUFA) fatty acids in zebrafish larvae, contribution of n3, n6 and n9 fatty acids to lipid profiles (as mg/g dry weight) and n6/n3 ratio. Different letters indicate statistically significant differences among experimental groups compared within the same fatty acid class ($p<0.05$). Values are presented as mean ± SD ($n = 3$). Zebrafish larvae fed diet based on fish meal (Control) and zebrafish larvae fed diet with 25% (Group A) or 50% (Group B) replacement of fish meal with BSF meal.

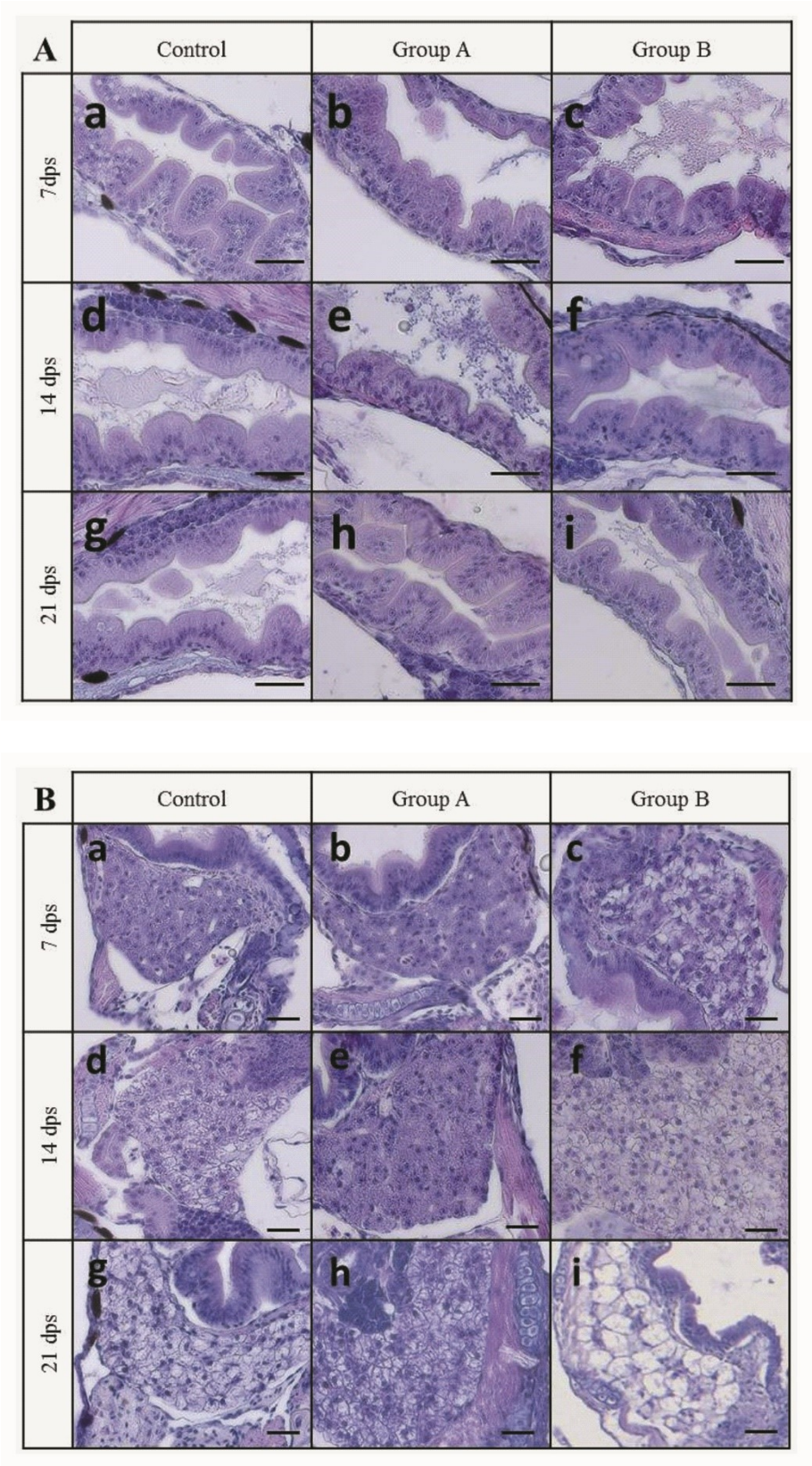

Fig. 5. Example of histomorphology (at 7,14 and 21 dps) of zebrafish: (A) intestine Control (a,d,g), Group A (b,e,h), Group B (c,f,i); (B) liver Control (a,d,g), Group A (b,e,h), Group B (c,f,i). Scale bars: 25 μm. Zebrafish larvae fed diet based on fish meal

(Control) and zebrafish larvae fed diet with 25% (Group A) or 50% (Group B) replacement of fish meal with BSF meal.

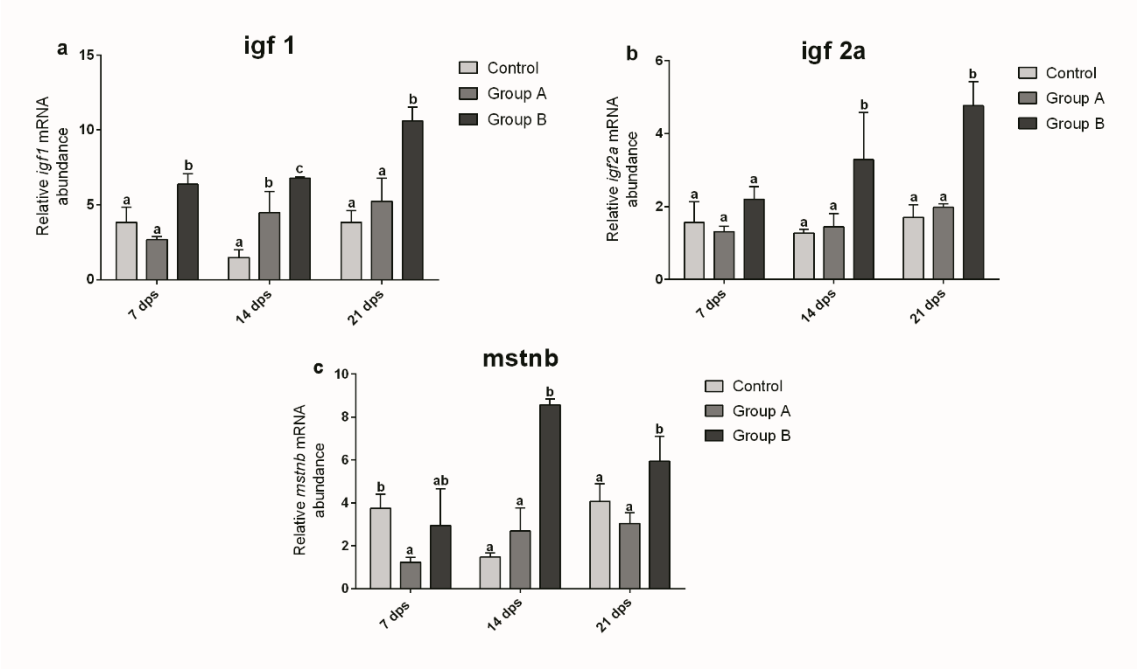

Fig. 6. Relative mRNA levels of genes involved in growth analyzed in zebrafish larvae from the three experimental groups at 7, 14 and 21 dps. (a) *igf1*, (b) *igf2a*, (c) *mstnb*. Different letters indicate statistically significant differences among experimental groups compared within the same sampling time ($p<0.05$). Values are presented as mean ± SD (n = 3). Zebrafish larvae fed diet based on fish meal (Control) and zebrafish larvae fed diet with 25% (Group A) or 50% (Group B) replacement of fish meal with BSF meal.

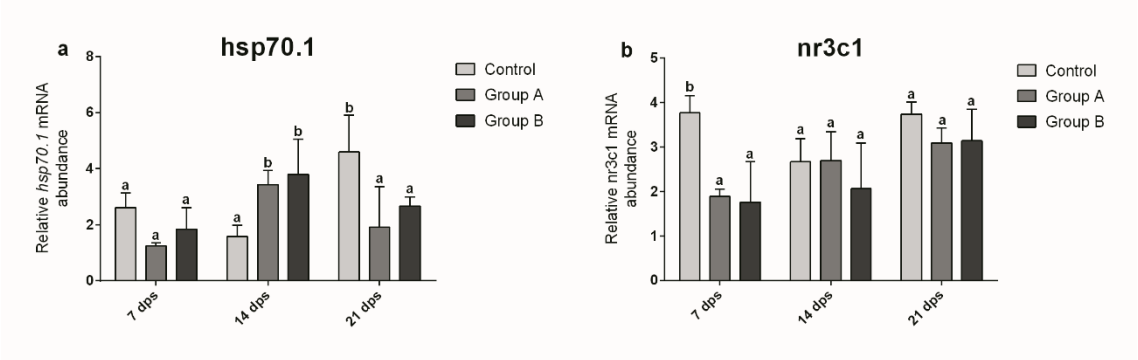

Fig. 7. Relative mRNA levels of genes involved in stress response analyzed in zebrafish larvae from the three experimental groups at 7, 14 and 21 dps. (a) *hsp70.1*, (b) *nr3c1*. Different letters indicate statistically significant differences among experimental groups compared within the same sampling time ($p<0.05$). Values are presented as mean ± SD (n = 3). Zebrafish larvae fed diet based on fish meal (Control) and zebrafish larvae fed diet with 25% (Group A) or 50% (Group B) replacement of fish meal with BSF meal.

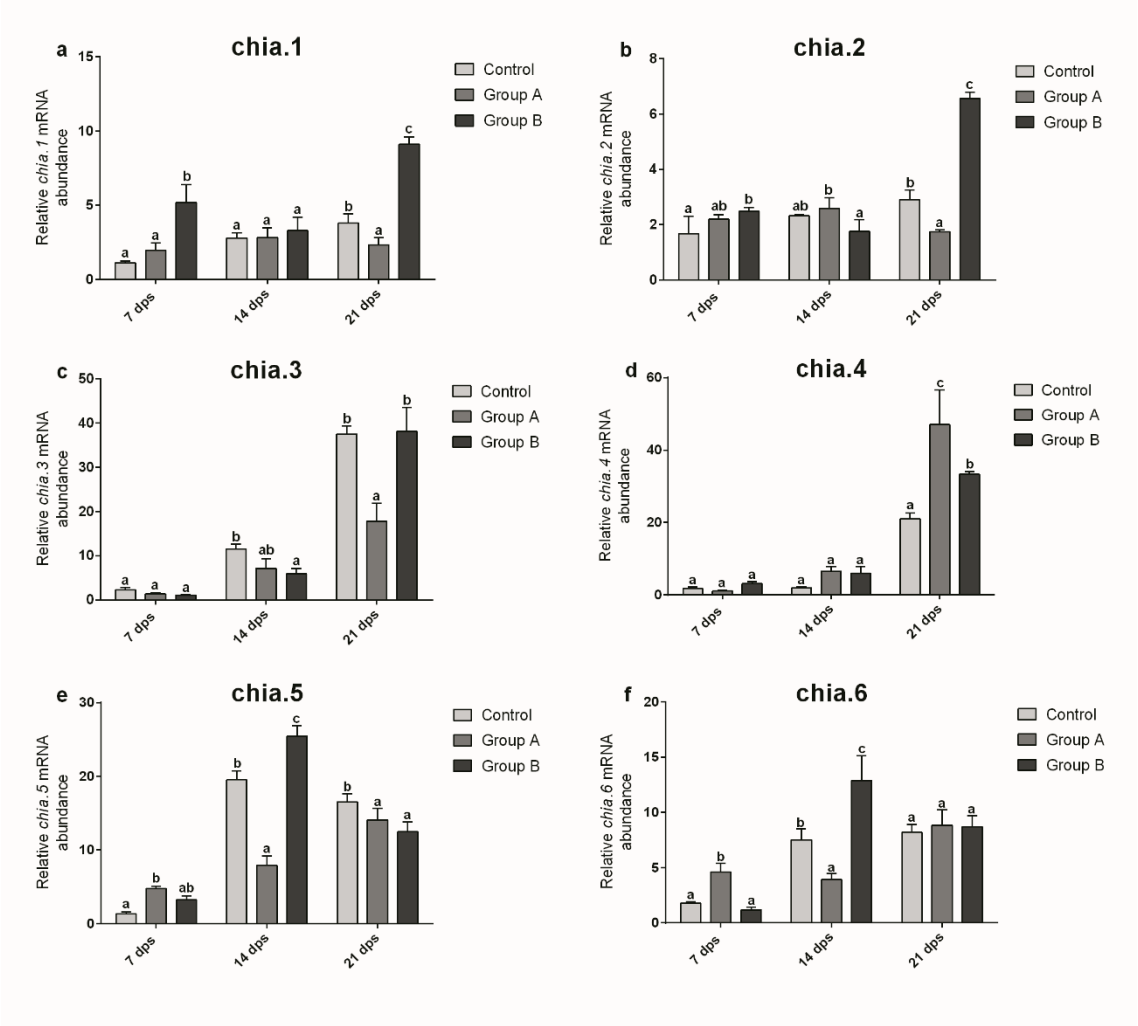

Fig. 8. Relative mRNA levels of genes involved in enzymatic hydrolysis of chitin analyzed in zebrafish larvae from the three experimental groups at 7, 14 and 21 dps. (a) *chia.1*, (b) *chia.2*, (c) *chia.3*, (d) *chia.4*, (e) *chia.5*, (f) *chia.6*. Different letters indicate statistically significant differences among experimental groups compared within the same sampling time ($p<0.05$). Values are presented as mean ± SD (n = 3). Zebrafish larvae fed

diet based on fish meal (Control) and zebrafish larvae fed diet with 25% (Group A) or 50% (Group B) replacement of fish meal with BSF meal.

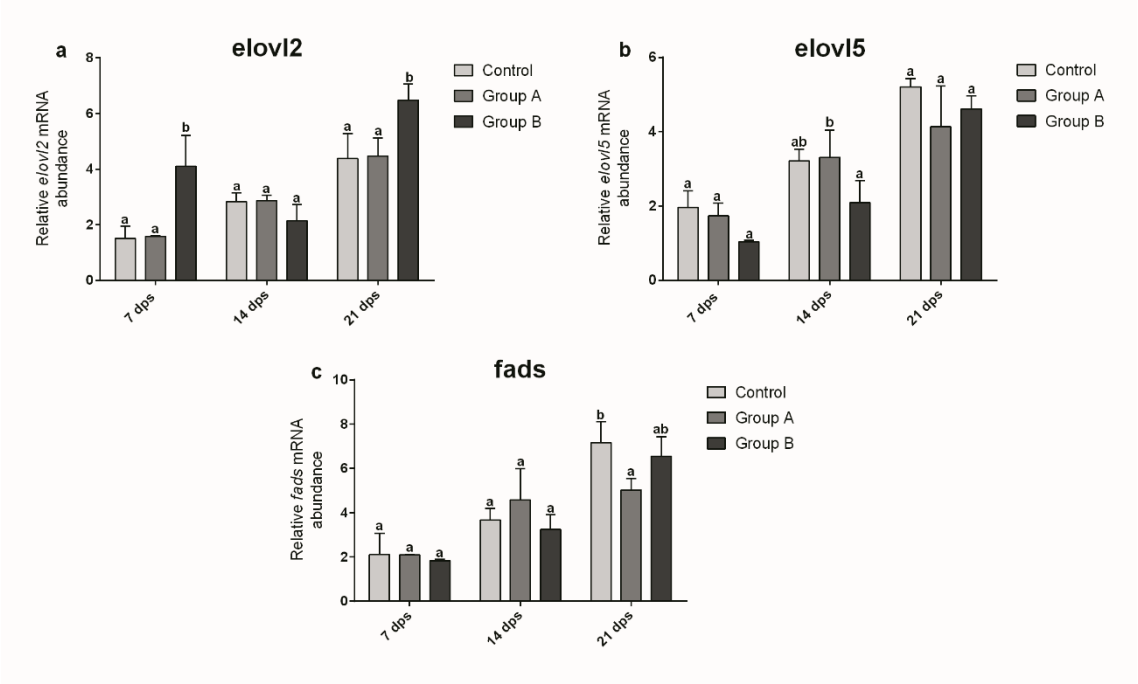

Fig. 9. Relative mRNA levels of genes involved in lipid metabolism and in the long-chain polyunsaturated fatty acids biosynthesis analyzed in zebrafish larvae from the three experimental groups at 7, 14 and 21 dps. (a) *elovl2*, (b) *elovl5*, (c) *fads2*. Different letters indicate statistically significant differences among experimental groups compared within the same sampling time ($p<0.05$). Values are presented as mean ± SD (n = 3). Zebrafish larvae fed diet based on fish meal (Control) and zebrafish larvae fed diet with 25% (Group A) or 50% (Group B) replacement of fish meal with BSF meal.

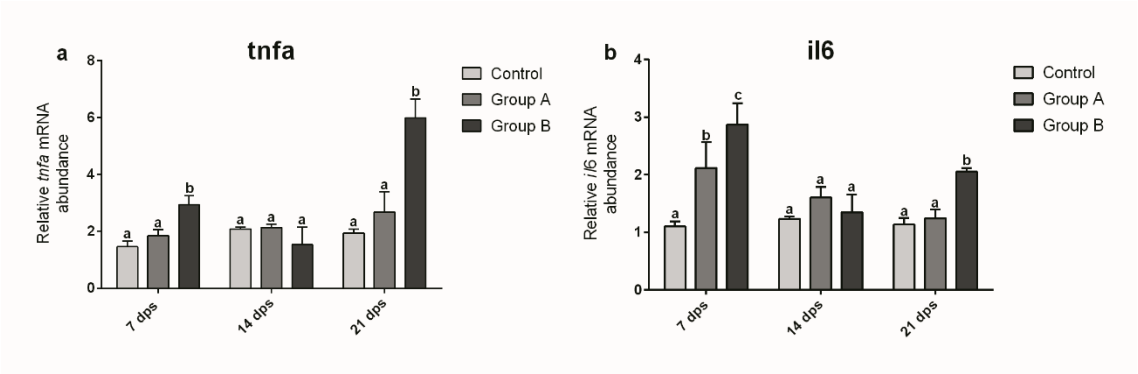

Fig. 10. Relative mRNA levels of genes involved in immune response analyzed in zebrafish larvae from the three experimental groups at 7, 14 and 21 dps. (a) *tnfa*, (b) *il6*. Different letters indicate statistically significant differences among experimental groups compared within the same sampling time ($p<0.05$). Values are presented as mean ± SD (n = 3). Zebrafish larvae fed diet based on fish meal (Control) and zebrafish larvae fed diet with 25% (Group A) or 50% (Group B) replacement of fish meal with BSF meal.

Chapter 3

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Insect meal based diets for clownfish: biometric, histological, spectroscopic, biochemical and molecular implications

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Abstract

Basic nutrition for tropical fish is undeniably lacking, mainly due to the concentration of research efforts on and funding for food fish species. However, as the marine ornamental aquaculture industry continues to grow and expand, it is now essential to direct nutritional research to mitigate the current lack of information related to feeds specifically formulated for ornamental fish. Due to a minimal environmental impact, compared to most conventional feed commodities, insects deserve a growing attention as candidate ingredient for aquafeeds and *Hermetia illucens*, the black soldier fly, is presently one of the most promising insect species. The aim of the present study was to formulate new insect based diets and to evaluate, through a multidisciplinary approach including morphological, biochemical, spectroscopic and molecular analysis, the major biological responses of *Amphiprion ocellaris* juveniles fed on these diets over a 106 days experimental period. Generally, results were promising since no negative effects on fish growth, stress response and survival were detected. However, the dietary inclusion of black soldier fly affected the fatty acid composition of the fish.

Keywords

Black soldier fly, gene expression, fatty acid profile, insect diet, ornamental aquaculture, clownfish, FTIRM.

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1. Introduction

The ornamental fish industry is a worldwide economically important activity valued at approximately US$15 billion (Bartley, 2000; Rhyne et al., 2017). For many years, it has exclusively relied on the supply of animals collected from the wild, often using destructive fishing methods such as cyanide and dynamite (Wabnitz et al., 2003; Olivotto et al., 2010, 2017; Calado et al., 2017). Today, less than 10 percent of marine aquarium fish are captive-bred, as opposed to greater than 90 percent of the fresh water species (Olivotto et al., 2003; Calado et al., 2017). Consequently, there is a need to develop a marine ornamental aquaculture (Olivotto et al., 2017). This sector still faces several problems related to broodstock management, spawning induction, embryo development, hatching and the development of efficient grow out diets (Olivotto et al., 2008, 2017; Randazzo et al., 2018; Vargas-Abúndez et al., 2018).

Basic nutrition for tropical fish is undeniably lacking, mainly due to the concentration of research efforts on and funding for food fish species. There is a disproportionate level of research dedicated to the aquaculture production of food fish when compared to the ornamental industry. Current diets commercially available for ornamental fish are generally too expensive for large-scale procedures (Calado et al., 2017). As the marine ornamental aquaculture industry continues to grow and expand, it will be essential to

direct nutritional research to mitigate the current lack of information related to feeds formulated specifically for ornamental fish (Calado et al., 2017).

The need for more profitable and sustainable aquafeed ingredients has already been explored for finfish production (Oliva-Teles et al., 2015; Gasco et al., 2018a) in order to replace expensive and unsustainable fish meal (FM) and fish oil (Tacon and Metian, 2015; Gasco et al., 2018a). Research to develop substitutes for these feed ingredients has focused on commodities such as oilseeds (especially soybeans), meat by-products (such as blood meal and bone meal) and microbial proteins (Francis et al., 2001; Tibaldi et al., 2015; Gasco et al., 2018a).

However, it should be pointed out that complete replacement of FM and fish oil in finfish aquaculture feeds faced severe barriers (Francis et al., 2001; Tibaldi et al., 2015; Gasco et al., 2018a). Especially for carnivorous fish, vegetable proteins showed inappropriate amino-acid balance, poor protein digestibility and antinutritional substances (Francis et al., 2001; Chong et al., 2003; Gai et al., 2012; Gasco et al., 2018a). Therefore, inclusion of other highly nutritious supplements such as microalgae (Tulli et al., 2012; Tibaldi et al., 2015) and/or meat by-products (Gasco et al., 2018a) have been explored; however, these ingredients do not always meet the expected ecological, nutritional and economical requirements.

As a consequence both finfish and ornamental aquaculture could benefit of cheaper, environmentally friendly and nutritious alternative ingredients (Chong et al., 2003; Alencastro et al., 2005). Alternatives must ensure fish health and welfare standards by providing proper feeding stimulants (Xue and Cui, 2001), proper levels of indispensable amino acids and polyunsaturated fatty acids (PUFAs) (Barroso et al., 2017), high nutrient and energy bioavailability (Gasco et al., 2018a), and reduced antinutritional factors (Gai et al., 2012). Due to a minimal environmental impact, compared to most conventional

feed commodities, insects deserve a growing attention as candidate ingredient for aquafeeds. They are cultured through environmental-friendly, cost-effective farming processes, since they can be produced on by-products/wastes (Barroso et al., 2014; van Huis and Tomberlin, 2017; Meneguz et al., 2018; Vargas et al., 2018). They are rich in proteins, with an essential amino acid composition similar to that of FM, and in lipids (Belforti et al., 2015; Henry et al., 2015; Roncarati et al., 2015; Barroso et al., 2017; Devic et al., 2017; Iaconisi et al., 2017; Paul et al., 2017; Belghit et al., 2018). *Hermetia illucens*, the black soldier fly (BSF), is presently one of the most promising insect species for the aquaculture industry (Henry et al., 2015; Renna et al., 2017) and preliminary results are promising (Borgogno et al., 2017; Renna et al., 2017; Belghit et al., 2018; Bruni et al., 2018; Vargas et al., 2018; Zarantoniello et al., 2018).

If insects may represent an economically and ecologically sound ingredient for both finfish and ornamental aquafeed, their use still faces possible limitations including their poor PUFAs content (Barroso et al., 2017; Paul et al., 2017), and the presence of chitin (Kroeckel et al., 2012; Magalhães et al., 2017; Su et al., 2017; Xiao et al., 2018).

The false percula clownfish, *Amphiprion ocellaris,* aside being a very popular aquarium fish, represents an ideal marine experimental model since it can be easily kept in laboratory conditions, spawns regularly and its genome is partially available in GenBank (Avella et al., 2009; Olivotto et al., 2010, 2011; Marcionetti et al., 2017).

Because of the high prices of ornamental fish feeds and the need of more sustainable and ecologically sound ingredients, the aim of the present study was to formulate new BSF based diets and to evaluate, through a multidisciplinary approach including morphological, biochemical, spectroscopic and molecular analysis, the major biological responses of *A. ocellaris* juveniles fed on these diets.

2. Materials and Methods

2.1. Ethics

All procedures involving animals were conducted in line with Italian legislation on experimental animals. 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 anesthetic (MS222; Sigma Aldrich).

2.2. Insect source

BSF larvae meal was purchased from Hermetia Deutschland GmbH & Co. KG (Baruth/Mark, Germany). BSF larvae meal was partially defatted with a mechanical process performed using high pressure and without solvents. No other information was provided by the producer on substrate or processing methodologies, as they are considered confidential.

2.3. Fish and experimental conditions

Juvenile clownfish (*Amphiprion ocellaris*, Pomacentridae) were purchased from a pet shop (La Casetta in Canadà, SettimoTorinese, IT) and maintained in a 200 L tank for 15 days. Seawater was treated with mechanical, biological and UV filtration (Panaque, Italy; Olivotto et al., 2002; Randazzo et al., 2017). Water temperature was maintained at 28°C, salinity at 30‰, pH at 8–8.5, NH_3 and NO_2^- < 0.02 mg/L and NO_3^- < 5 mg/L. Photoperiod was maintained at 13 h light/11 h dark (Olivotto et al., 2004). Fish were fed *ad libitum* twice a day with a commercial feed (EscheMatteo, Parma, Italy) and *Artemia* nauplii (INVE, Belgium).

2.4. Feed formulation

Four experimental diets were formulated at the Department of Agricultural, Forest and Food Sciences, University of Turin, Italy. They were obtained including graded levels (0, 20, 40 and 60%) of defatted BSF larvae meal (BSF larvae meal substituted 0% (HI0), 25% (HI25), 50% (HI50) and 75% (HI75) of FM). All ingredients were thoroughly mixed; water was then blended into the mixture to attain an appropriate consistency for pelleting using a 1 mm die meat grinder. After pelleting, the diets were dried at 40 °C and stored in plastic bags at -20 °C until used. The ingredients and proximate composition of the experimental diets are reported in Table 1.

Table 1. Ingredients and proximate composition of experimental diets.

	HI0	HI25	HI50	HI75
Ingredients (g kg^{-1})				
Fish meal	600.0	450.0	300.0	150.0
HI larvae meal	0.0	200.0	400.0	600.0
Seeds meal	20.0	20.0	20.0	20.0
Wheat meal	90.0	75.0	60.0	45.0
Fish oil	110.0	75.0	40.0	5.0
Starch	150.0	150.0	150.0	150.0
Vit	10.0	10.0	10.0	10.0
Min	10.0	10.0	10.0	10.0
DL methionine	5.0	5.0	5.0	5.0
Lysine	5.0	5.0	5.0	5.0
Proximate composition				
Dry matter (g 100 g^{-1})	95.2	95.4	94.2	95.5
Crude protein (g 100 g^{-1}, as fed)	48.5	49.0	49.4	50.3
Ether extract (g 100 g^{-1}, as fed)	16.9	12.9	10.1	6.5
Ash (g 100 g^{-1}, as fed)	10.6	9.2	7.8	6.4
Nitrogen free extract (g 100 g^{-1}, as fed)	17.7	21.4	25.1	28.8

2.5. Experimental design

Fish were divided in twelve 150 L experimental tanks (3 tanks per dietary group) (25 fish in each tank) that were randomly assigned to one of the four dietary treatments (each in triplicate).

Clownfish were fed the experimental diets at 2 % body weight, twice a day. Water characteristics were maintained as described above. Fish were weighted at the beginning of the experiment (T0) and after 106 days of dietary treatment (T1). Samplings, for further analyses, were performed at the end of the experiment and fish were euthanized with an excess of anesthetic (MS222 1g/L, Sigma; Piccinetti et al., 2012).

2.6. Biometry

At the beginning and at the end of the experiment, five fish, in triplicate, were randomly collected from the different tanks of each experimental group. The wet weight was estimated with an OHAUS Explorer analytical balance (precision 0.1 mg) and the specific growth rate (SGR) for each experimental group was calculated as follows:

$SGR\% = ((\ln Wf - \ln Wi)/t) \times 100$,

where *Wf* is the final wet weight, *Wi*, the initial wet weight, and *t*, the number of days (106).

Finally, the feed conversion ratio (FCR) for each experimental group was calculated as:

FCR = total feed fed (g)/total wet weight gain (g).

2.7. Lipid extraction and fatty acid analysis

Experimental diets (in triplicate) and whole fish (three fish per tank –in total 9 fish per experimental group at the end of the experiment) were minced, homogenized (MZ 4110, DCG Eletronic) and freeze-dried (Edwards EF4, Crawley, Sussex, England). Lipid extraction was carried out on lyophilized powders following a Microwave-Assisted Extraction (MAE) (Illuminati et al., 2010; Truzzi et al., 2017). Fatty acid methyl esters (FAMEs) were prepared according to Truzzi et al. (2017), using the methyl ester of nonadecanoic acid (19:0, Dr. Ehrenstorfer GmbH, Germany) as internal standard. FAMEs

were determined on an Agilent-6890 GC System coupled to an Agilent-5973N quadrupole Mass Selective Detector (MSD). A CPS ANALITICA CC-wax-MS (30 m × 0.25 mm ID, 0.25 μm film thickness) capillary column was used to separate FAMEs. Instrumental conditions were set up as in Truzzi et al. (2018), and no overlapping between peaks were noted. For each sample, at least three runs were performed on the GC-MS. The precision of the proposed method, the limit of detection and the limit of quantification were evaluated for the studied matrices and they were similar to those found for fish muscle (Truzzi et al., 2017).

2.8. Histology

Nine clownfish randomly collected at the end of the experiment from the different tanks (3 per tank), belonging to the dietary treatments, were dissected to remove the liver and intestines. The liver and intestine samples were fixed by immersion in 4 % paraformaldehyde and stored at 4 °C for 24 h. Samples were washed 3 times with PBS 0.1 M (pH 7.4) for ten minutes and preserved in ethanol (70 %). After dehydration by subsequent washing in ethanol (80, 95 and 100 %), samples were washed with clearing agent "Histo-Clear" (Bio-Clear, Bio Optica) and embedded in paraffin (Bio-Optica, Milano, Italy). Solidified paraffin blocks were cut with a microtome (Leica RM2125 RTS) and 5 μm sections were stained with Mayer hematoxylin and eosin Y (Sigma-Aldrich). Additionally, liver sections were stained using periodic acid of Schiff reaction (PAS) following the manufacturer's instructions (BIO-OPTICA). Adjacent sections were treated with α-amilase before PAS staining in order to be sure that staining results were exclusively related to glycogen content. For the morphometric analysis of intestine folds, three histological sections at ~200μm intervals were analyzed from each fish belonging to the different dietary treatments. Measurements were performed on the medium tract of

the intestine and folds length was measured starting from their base to the apical tip (Falcinelli et al., 2015). Sections were observed using a Leica MD750 optical microscope connected with a camera Leica ICC50 HD.

2.9. Fourier Transform Infrared Microspectroscopy (FTIRM) measurements and data analysis

Nine clownfish were randomly collected for each experimental group (3 per tank) at the end of the experiment. Fish were euthanized and dissected to remove the liver. Livers were immediately cryopreserved at -80°C until cutting. From each liver sample, 5 thin sections (~10 μm thick) were cut at 100 μm intervals with a cryomicrotome (Microm HM 505 N). Sections were deposited, without any fixation process, onto CaF_2 optical windows (1 mm thick, 13 mm diameter), and air-dried for 30 min (Giorgini et al., 2015). FTIRM measurements were carried out within 48 hours from cutting. Similar samples prepared by using the same protocol were already tested in our laboratory, evidencing a good stability in time and providing homogeneous and reliable vibrational data sets. A Perkin Elmer Spectrum GX1 Spectrometer interfaced with a AutoImage microscope (6x condenser objective) and equipped with a photoconductive 0.25 mm Hg-Cd-Te (MCT) array detector, operating at liquid nitrogen temperature and covering the entire IR spectral range from 4000 to 700 cm^{-1}, was adopted. By using a 15× condenser/objective, the photomicrograph of each section was collected. Then, some areas (~1000x1000 μm^2), considered representative of the sample were selected, on which the IR maps were acquired in transmission mode and at room temperature, in the MIR range from (4000 - 800 cm^{-1}, spectral resolution 4 cm^{-1}). IR maps are false color images representing the topographical distribution of the total absorption of the infrared radiation on the mapped area; each pixel corresponds to a IR spectrum. Due to the nature of liver, which can be

considered almost homogeneous, and to acquire larger areas, a spatial resolution of 40x40 μm^2 was adopted. On each section, a background spectrum was acquired on a clean portion of the CaF_2 optical window. IR maps were vector normalized on the full frequency range (to avoid artifacts due to local thickness variations) (Spectrum Autoimage 5.1.0 software package, Perkin Elmer, Waltham, MA, USA).

The topographic distribution of the most representative tissue components was obtained by integrating IR maps under the following spectral regions: 3000-2824 cm^{-1} (representative of overall lipids, named LIP); 1775-1723 cm^{-1} (representative of overall fatty acids, named FA); 1723-1488 cm^{-1} (representative of overall proteins, named PRT), and 1074-963 cm^{-1} (representative of overall glycogen, named GLY). An arbitrary color scale was used, white color indicating the pixels with the highest absorbance values, whilst blue color representing those with the lowest ones.

To obtain more information on the biochemical composition of liver samples, the IR spectra of each map were vector normalized in the 3800-950 cm^{-1} spectral range, and then the integrated areas of the following spectral regions calculated (Spectrum 10.4 software package, Perkin Elmer, Waltham, MA, USA): 3000-2824 cm^{-1} (symmetric and asymmetric stretching modes of CH_2 and CH_3 groups in lipid alkyl chains, representative of lipids, named LIP); 1775-1723 cm^{-1} (stretching mode of CO ester moiety of fatty acids, named FA); 1723-1488 cm^{-1} (Amide I and Amide II bands of proteins, representative of proteins, named PRT); 1488-1430 cm^{-1} (bending modes of CH_2 groups in lipid alkyl chains, representative of saturated alkyl chains, named CH_2), and 1074-963 cm^{-1} (vibrational modes of carbohydrates mostly attributed to glycogen, named GLY). The sum of the integrated areas of the regions 3000–2824 cm^{-1} and 1800–950 cm^{-1} was considered representative of the overall tissue biomass (named TBM). The integrated areas were used to calculate the following band area ratios: LIP/TBM (relative amount of

lipids); FA/TBM (relative amount of fatty acids); CH_2/TBM (relative amount of saturated alkyl chains); CH_2/LIP (relative amount of saturated alkyl chains with respect to lipids); PRT/TBM (relative amount of proteins); GLY/TBM (relative amount of glycogen), GLY/PRT (relative amount of glycogen with respect to proteins) and GLY/LIP (relative amount of glycogen with respect to lipids).

For all experimental groups, the average absorbance spectra were also calculated in the spectral range from 3800 cm^{-1} to 950 cm^{-1} and converted in second derivative mode, to retrieve the position of the most relevant absorption bands. The vibrational and biological assignments of each absorption band were performed according to the literature.

2.10. RNA extraction and cDNA synthesis

Total RNA extraction from single clownfish livers (five replicates per dietary treatment randomly collected at the end of the experiment from HI0, HI25, HI50 and HI75 tanks), was optimized using RNAzol® RT reagent (SIGMA-ALDRICH®, R4533) following the manufacturer's instructions. RNA was then eluted in 20 µl of RNase-free water. Final RNA concentration was determined by the NanoPhotometer® P-Class (Implen, 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, 3 µg of RNA was used for cDNA synthesis, employing the High Capacity cDNA Reverse Transcription Kit (Bio-Rad) following the manufacturer's instructions.

2.11. Real-Time PCR

PCRs were performed with SYBR Green in an iQ5 iCycler thermal cycler (both from Bio-Rad) in triplicate. Reactions were set on a 96-well plate by mixing for each sample 1 µL cDNA diluted 1:20, 5 µL of 2x concentrated iQ™ Sybr Green as the fluorescent

intercalating agent, 0.3 µM forward primer, and 0.3 µM 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 60 °C and 20 s at 72 °C. Fluorescence was monitored at the end of each cycle. Dissociation curve analysis showed a single pick in all cases.

Relative quantification of the expression of genes involved in fish growth (*igf1*, *igf2* and *mstn*), lipid metabolism (*pparα* and *pparβ*) and stress response (*gr* and *hsp70*) was performed using *βactin* as housekeeping gene to standardize the results (Table 2).

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 analyzed 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 is reported with respect to controls. For making primers for real time PCR, sequenced regions of target genes for several species closely related to clownfish as well as outgroup species were aligned and the consensus sequences were used. Primers were used at a final concentration of 10 pmol/µL.

Table 2. List of primers used in this study.

Gene	Forward primer (5'- 3')	Reverse primer (5'- 3')
igf1	TGTCTAGCGCTCTTTCCTTTCA	AGAGGGTGTGGCTACAGGAGATAC
igf2	CGGCAGAAACGCTATGTGGA	TGCTGGTTGGCCTACTGAAA
mstn	TGAACAAAGCCAACCCCAGA	TCAAGAGCATCCACAACGGT
pparα	TTCAGCGACATGATGGAGCC	CAGTTTCTGCAGCAGATTGG
pparβ	AGGAGATAGGGGTACACGTG	CAGGAACTCCCGGGTCACAA
gr	CGGTCACTGCTACGTCTTCA	CCTCCCAGCACACAGGTAAT
hsp70	ACGGAGAGTCGATTTCGATG	GAAGGACATCAGCGACAACA
βactin	GGTACCCATCTCCTGCTCCAA	GAGCGTGGCTACTCCTTCACC

2.12. Statistics

All data were analyzed by one way ANOVA, with diet as the explanatory variable. All ANOVA tests were followed by Tukey's post-test. The statistical software package Prism5 (GraphPad Software) was used; significance was set at $P < 0.05$. All results are presented as mean ± S.D.

3. Results

3.1. Biometry

Considering SGR, no significant differences ($P > 0.05$) were observed among experimental groups; HI0 showed 1.2 ± 0.2 % day^{-1} while HI25, HI50 and HI75 showed 1.0 ± 0.2, 1.0 ± 0.3 and 1.1 ± 0.1 % day^{-1}, respectively (Fig. 1a). As regards FCR, no significant differences ($P > 0.05$) among the experimental groups were detected: HI0 showed 4.6 ± 1.0 FCR while HI25, HI50 and HI75 showed 5.2 ± 0.4, 4.5 ± 0.9 and 4.5 ± 1.2 FCR, respectively (Fig. 1b).

3.2. Fatty acid composition of experimental diets

Table 3 shows the fatty acid composition of the experimental diets. Considering FA classes, the percentage variation was strongly influenced by the percentage of BSF substitution in the diets. In particular, total SFA percentage significantly increased ($P < 0.05$) with the increase of BSF inclusion, with SFA percentage ranging from 7.6 ± 0.3% in HI0 diet to 11.2 ± 0.2%, 16.7 ± 0.3% and 34.4 ± 0.7% in HI25, HI50 and HI75 diets, respectively. Total MUFA percentage, on the contrary, significantly decreased ($P < 0.05$) in HI50 (45 ± 0.4%) and HI75 (36.2 ± 0.3%) diets with respect to HI0 diet (49.8 ± 0.6%). Total PUFA percentage showed a statistically significant decrease ($P < 0.05$) in HI25 (39.2 ± 0.6%), HI50 (38.3 ± 0.4%) and HI75 (29.4 ± 0.5%) diets with respect to HI0 diet (42.6 ± 0.7%). Total n3 FAs significantly decreased ($P < 0.05$) with the increase of BSF

inclusion (38.3 ± 0.7%, 34.3 ± 0.6%, 32.6 ± 0.4% and 20.4 ± 0.5% in HI0, HI25, HI50 and HI75 diets, respectively). As a consequence, the n3/n6 ratio significantly decreased ($P < 0.05$) with the increase of BSF inclusion (8.9 ± 0.2%, 7.1 ± 0.2%, 5.7 ± 0.1% and 2.3 ± 0.1 in HI0, HI25, HI50 and HI75 diets, respectively).

Specifically, as regards SFAs, lauric (12:0), myristic (14:0) palmitic (16:0) and stearic acids (18:0) significantly increased ($P < 0.05$) with the increase of BSF meal inclusion, (Table 3), with lauric acid ranging from 0.03 ± 0.01% in HI0 diet to 2.1 ± 0.1%, 5.2 ± 0.06% and 14 ± 0.3% in HI25, HI50 and HI75 diets, respectively; with myristic acid ranging from 1.5 ± 0.1% in HI0 diet to 2.1 ± 0.1%, 3.1 ± 0.1% and 6.5 ± 0.1% in HI25, HI50 and HI75 diets, respectively; with palmitic acid ranging from 4.1 ± 0.1% in HI0 to 4.8 ± 0.2%, 6.1 ± 0.1% and 10 ± 0.1% in HI25, HI50 and HI75 diets, respectively; and with stearic acid ranging from 1.4 ± 0.1% in HI0 to 1.5 ± 0.1%, 1.7 ± 0.1% and 2.3 ± 0.1% in HI25, HI50 and HI75 diets, respectively.

As regards MUFAs, the most represented FAs in HI0 diet were oleic (18:1n9; 17 ± 0.2%), eicosenoic (20:1n9; 12.8 ± 0.1%) and erucic acids (22:1n9; 9.5 ± 0.1%). Oleic acid showed a statistically significant increase ($P < 0.05$) with the increase of BSF inclusion ranging from 17 ± 0.2% in HI0 to 18 ± 0.4%, 18 ± 0.2% and 20 ± 0.2% in HI25, HI50 HI75 diets, respectively, while eicosenoic and erucic acids showed an opposite trend ($P > 0.05$), with eicosenoic acid ranging from 12.8 ± 0.1% in HI0 to 12.0 ± 0.1% 8.7 ± 0.1% and 3.2 ± 0.1% in HI25, HI50 HI75 diets, respectively, and with erucic acid ranging from 12.8 ± 0.1% in HI0 to 12.0 ± 0.1%, 8.7 ± 0.1% and 3.2 ± 0.1% in HI25, HI50 HI75 diets, respectively.

As regards PUFAs, the most represented FAs in HI0 diet were docosahexaenoic (22:6n3; 22.1 ± 0.4%) and eicosapentaenoic acid (20:5n3; 14.6 ± 0.1%). They showed a statistically significant decrease ($P < 0.05$) with the increase of BSF inclusion in the diets,

with docosahexaenoic acid ranging from 22.1 ± 0.4% in HI0 to 19.3 ± 0.6%, 18.6 ± 0.51% and 11.7 ± 0.9% in HI25, HI50 HI75 diets, respectively, and eicosapentaenoic acid ranging from 14.6 ± 0.1% in HI0 to 13.3 ± 0.2%, 12.2 ± 0.3% and 6.7 ± 0.2% in HI25, HI50 HI75 diets, respectively. Linoleic acid (18:2n6), on the contrary, showed a statistically significant increase ($P < 0.05$) with the increase of BSF inclusion in the diet ranging from 2.4 ± 0.1% in HI0 diet to 3.0 ± 0.1%, 4.1 ± 0.1% and 8.0 ± 0.1% in HI25, HI50 and HI75 diets, respectively.

Table 3. Fatty acid composition (as percentage of total FAs; only FAs > 1% of total FAs) of experimental diets. For each matrix, means within rows bearing different letters are significantly different ($P < 0.05$).

FAs	HI0	HI25	HI50	HI75
12:0	0.03 ± 0.01^{a}	2.1 ± 0.1^{b}	5.2 ± 0.1^{c}	14.4 ± 0.3^{d}
14:0	1.5 ± 0.1^{a}	2.1 ± 0.1^{b}	3.1 ± 0.1^{c}	6.5 ± 0.1^{d}
16:0	4.1 ± 0.1^{a}	4.8 ± 0.2^{b}	6.1 ± 0.1^{c}	10.2 ± 0.1^{d}
16:1n9	5.6 ± 0.1^{a}	6.0 ± 0.1^{b}	6.3 ± 0.1^{c}	7.1 ± 0.1^{c}
18:0	1.4 ± 0.1^{a}	1.5 ± 0.1^{a}	1.7 ± 0.1^{a}	2.3 ± 0.1^{b}
18:1n9	16.7 ± 0.2^{a}	18.0 ± 0.4^{b}	18.2 ± 0.2^{b}	19.8 ± 0.2^{c}
18:1n7	3.1 ± 0.1^{a}	3.0 ± 0.1^{a}	2.7 ± 0.1^{a}	1.7 ± 0.1^{b}
18:2n6	2.4 ± 0.1^{a}	3.0 ± 0.1^{b}	4.1 ± 0.1^{c}	8.0 ± 0.1^{d}
18:3n3	1.3 ± 0.1^{a}	1.4 ± 0.1^{a}	1.5 ± 0.1a^{b}	1.9 ± 0.1^{b}
20:1n9	12.8 ± 0.1^{a}	12.0 ± 0.1^{b}	8.7 ± 0.1^{c}	3.2 ± 0.1^{d}
20:4n6	1.3 ± 0.1^{a}	1.2 ± 0.1^{a}	1.1 ± 0.1^{ab}	0.7 ± 0.1^{b}
20:5n3	14.6 ± 0.1^{a}	13.3 ± 0.2^{b}	12.2 ± 0.3^{c}	6.7 ± 0.2^{d}
22:1n9	9.5 ± 0.1^{a}	8.7 ± 0.1^{b}	7.1 ± 0.1^{c}	2.2 ± 0.1^{d}
22:6n3	22.1 ± 0.4^{a}	19.3 ± 0.6^{b}	18.6 ± 0.51^{c}	11.7 ± 0.9^{d}
24:1n9	1.6 ± 0.1^{a}	1.3 ± 0.1^{a}	1.3 ± 0.2^{a}	1.3 ± 0.3^{a}
Total SFA+	7.6 ± 0.3^{a}	11.2 ± 0.2^{b}	16.7 ± 0.3^{c}	34.4 ± 0.7^{d}
Total MUFA*	49.8 ± 0.6^{a}	49.7 ± 0.9^{a}	45.0 ± 0.4^{c}	36.2 ± 0.3^{d}
Total PUFA#	42.6 ± 0.7^{a}	39.2 ± 0.6^{b}	38.3 ± 0.4^{b}	29.4 ± 0.5^{c}
Total n6^	4.3 ± 0.1^{a}	4.8 ± 0.1^{b}	5.7 ± 0.1^{c}	9.0 ± 0.1^{d}
Total n3	38.3 ± 0.7^{a}	34.3 ± 0.6^{b}	32.6 ± 0.4^{c}	20.4 ± 0.5^{d}
n6/n3	0.11 ± 0.01^{a}	0.14 ± 0.01^{a}	0.17 ± 0.01^{a}	0.44 ± 0.01^{b}
n3/n6	8.9 ± 0.2^{a}	7.1 ± 0.2^{b}	5.7 ± 0.1^{b}	2.3 ± 0.1^{c}
n9	46.1 ± 0.6^{a}	46 ± 0.9^{a}	41.6 ± 0.4^{b}	33.6 ± 0.3^{c}

$^{+}$includes also 10:0, 13:0, 15:0, 17:0, 20:0, 21:0, 22:0 and 23:0
*includes also 14:1n5, 15:1n5 and 17:1n7
$^{\#}$includes also 16:2n7, 18:3n6, 20:2n6 and 20:3n6

^includes also 18:3n6, 20:2n6 and 20:3n6

3.3. Fish lipid content

Both fish dry weight percentage and lipid percentage did not show any statistically significant difference ($P > 0.05$) among experimental groups (Table 4).

	HI0	HI25	HI50	HI75
% dry weight	29.5 ± 1.8[a]	25.4 ± 3.3[a]	25.2 ± 5.4[a]	25.5 ± 2.8[a]
% lipid dw	11.8 ± 3.9[a]	10.7 ± 1.3[a]	11.3 ± 3.4[a]	10.3 ± 1.1[a]

Table 4. Dry weight and lipid (dry matter basis) percentage in *A. ocellaris* juveniles fed diets where FM was substituted by 0 % (HI0), 25 % (HI25), 50 % (HI50) and 75 % (HI75) BSF meal, collected at the end of the experiment (106 days). No significant differences among groups were detected ($P > 0.05$).

3.4. Fish fatty acid composition

Table 5 shows the fatty acid composition of clownfish sampled at the end of the experiment. The FA composition of clownfish was deeply influenced by the BSF inclusion in the diets. In particular, total SFA percentage significantly increased ($P < 0.05$) with the increase of BSF meal inclusion in the diets, with SFA percentage ranging from 22.2 ± 0.4% in HI0 fish to 25.9 ± 0.4%, 30.3 ± 0.7% and 35.8 ± 1.0% in HI25, HI50 and HI75 fish, respectively. Total MUFA percentage, on the contrary, significantly decreased ($P < 0.05$) only in HI50 (30.6 ± 0.4%) and HI75 (28.5 ± 0.4%) fish with respect to HI0 fish (34.5 ± 0.4%). Total PUFA percentage showed a statistically significant decrease ($P < 0.05$) with the increase of BSF meal inclusion in the diet, ranging from 43.2 ± 0.6% in HI0 to 39.7 ± 0.2%, 39 ± 1.5% and 35.8 ± 0.8 in HI25, HI50 and HI75 fish, respectively. A similar trend was observed for total n3 FAs, with n3 ranging from 35.1 ± 0.6% in HI0 to 31.4 ± 0.2%, 29.7 ± 1.5% and 25.3 ± 0.8% in HI25, HI50 and HI75 fish, respectively.

As a consequence, the n3/n6 ratio significantly decreased ($P < 0.05$) with the increase of BSF inclusion, ranging from 4.5 ± 0.1% in HI0 to 3.9 ± 0.1%, 3.3 ± 0.2% and 2.6 ± 0.1% HI25, HI50 and HI75 fish, respectively.

Specifically, considering SFA in fish, lauric (12:0), myristic (14:0) and palmitic acid (16:0) percentage significantly increased ($P < 0.05$) with the increase of BSF meal inclusion in the diets, while stearic acid (18:0) did not show any significant difference ($P > 0.05$) among experimental groups (Table 5).

As regards MUFAs, the most represented FAs in HI0 fish were cis-7 hexadecenoic (16:1n9; 4.7 ± 0.1%), oleic (18:1n9; 14.0 ± 0.6%), eicosenoic (20:1n9; 6.5 ± 0.5%) and erucic (22:1n9; 3.7 ± 0.4%) acids. While cis-7 hexadecenoic acid and oleic acid did not show any significant difference ($P > 0.05$) among experimental groups, with cis-7 hexadecenoic acid ranging from 4.7 ± 0.1% in HI0 to 4.6 ± 0.6%, 4.7 ± 0.8% and 4.8 ± 0.1% in HI25, HI50 and HI75, respectively, and with oleic acid ranging from 14.0 ± 0.6% in HI0 to 14.7 ± 0.4%, 14.2 ± 0.9% and 14.6 ± 0.5% in HI25, HI50, HI75, eicosenoic and erucic acids, significantly decreased ($P < 0.05$) with the increase of BSF meal inclusion in the diets, with eicosenoic acid ranging from 6.5 ± 0.5 in HI0 to 6.4 ± 1.1%, 4.3 ± 0.9% and 3.0 ± 0.2% in HI25, HI50, HI75, respectively, and with erucic acid ranging from 3.7 ± 0.4% in HI0 to 3.4 ± 0.3%, 2.1 ± 0.7% and 1.0 ± 0.1% in HI25, HI50, HI75, respectively.

As regards PUFAs, the most represented FAs in HI0 fish were linoleic (18:2n6; 5.6 ± 0.2%), linolenic (18:3n3; 9.1 ± 0.5%), eicosapentaenoic (20:5n3; 5.3 ± 1.0%) and docosahexaenoic acids (22:6n3; 19.5 ± 1.1%). Linoleic and linolenic acids showed a statistically significant increase ($P < 0.05$) with the increase of BSF meal inclusion in the diets, with linoleic acid ranging from 5.6 ± 0.2% in HI0 to 6.0 ± 0.8%, 6.9 ± 0.6% and 7.8 ± 0.3% in HI25, HI50, HI75 fish, respectively, and with linolenic acid ranging from 9.1 ± 0.5% in HI0 to 7.1 ± 1.0%, 10.9 ± 0.6% and 11.4 ± 0.5% in HI25, HI50, HI75,

respectively. On the contrary, eicosapentaenoic and docosahexaenoic acids showed a statistically significant decrease ($P < 0.05$) with the increase of BSF meal inclusion in the diets, with eicosapentaenoic acid ranging from 5.3 ± 1.0% in HI0 to 5.1 ± 0.7%, 3.6 ± 0.9% and 2.7 ± 0.3% in HI25, HI50, HI75, and with docosahexaenoic acids ranging from 19.5 ± 1.1% in HI0 to 18.1 ± 0.5%, 13.6 ± 1.3% and 9.6 ± 0.7% in HI25, HI50, HI75.

Table 5. Fatty acid composition (as percentage of total FAs; only FAs > 1% of total FAs) of *A. ocellaris* juveniles fed diets where FM was substituted by 0 % (HI0), 25 % (HI25), 50 % (HI50) and 75 % (HI75) BSF meal, collected at the end of the experiment. For each matrix, means within rows bearing different letters are significantly different ($P < 0.05$).

FAs	HI0	HI25	HI50	HI75
12:0	0.1 ± 0.1^{a}	2.2 ± 0.1^{b}	4.0 ± 0.5^{c}	6.2 ± 0.4^{d}
14:0	3.0 ± 0.2^{a}	3.8 ± 0.4^{a}	4.6 ± 0.3^{b}	6.0 ± 0.3^{c}
16:0	12.3 ± 0.6^{a}	12.6 ± 0.3^{a}	13.9 ± 0.6^{b}	15.2 ± 0.2^{c}
16:1n9	4.7 ± 0.1^{a}	4.6 ± 0.6^{a}	4.7 ± 0.8^{a}	4.8 ± 0.1^{a}
18:0	4.8 ± 0.2^{a}	5.0 ± 0.4^{a}	5.3 ± 0.7^{a}	5.7 ± 0.2^{a}
18:1n9	14.0 ± 0.6^{a}	14.7 ± 0.4^{a}	14.2 ± 0.9^{a}	14.6 ± 0.5^{a}
18:1n7	3.5 ± 0.2^{a}	3.2 ± 0.1^{a}	3.1 ± 0.6^{a}	3.0 ± 0.2^{a}
18:2n6	5.6 ± 0.2^{a}	6.0 ± 0.8^{ab}	6.9 ± 0.6^{bc}	7.8 ± 0.3^{c}
18:3n3	9.1 ± 0.5^{a}	7.1 ± 1.0^{b}	10.9 ± 0.6^{c}	11.4 ± 0.5^{c}
20:1n9	6.5 ± 0.5^{a}	6.4 ± 1.1^{a}	4.3 ± 0.9^{b}	3.0 ± 0.2^{c}
20:4n6	1.0 ± 0.1^{a}	0.8 ± 0.1^{a}	0.8 ± 0.1^{a}	0.7 ± 0.1^{a}
20:3n3	1.2 ± 0.2^{a}	1.1 ± 0.3^{a}	1.6 ± 0.7^{a}	1.5 ± 0.3^{a}
20:5n3	5.3 ± 1.0^{a}	5.1 ± 0.7^{a}	3.6 ± 0.9^{b}	2.7 ± 0.3^{b}
22:1n9	3.7 ± 0.4^{a}	3.4 ± 0.3^{a}	2.1 ± 0.7^{b}	1.0 ± 0.1^{b}
22:6n3	19.5 ± 1.1^{a}	18.1 ± 0.5^{b}	13.6 ± 1.3^{c}	9.6 ± 0.7^{d}
Total SFA^{+}	22.2 ± 0.4^{a}	25.9 ± 0.4^{b}	30.3 ± 0.7^{c}	35.8 ± 1.0^{d}
Total MUFA*	34.5 ± 0.4^{a}	34.5 ± 0.4^{a}	30.6 ± 0.4^{b}	28.5 ± 0.4^{c}
Total PUFA$^{\#}$	43.2 ± 0.6^{a}	39.7 ± 0.2^{b}	39 ± 1.5^{b}	35.8 ± 0.8^{c}
Total n6^	7.8 ± 0.1^{a}	8.1 ± 0.1^{a}	9.1 ± 0.1^{b}	9.9 ± 0.2^{c}
Total n3	35.1 ± 0.6^{a}	31.4 ± 0.2^{b}	29.7 ± 1.5^{b}	25.3 ± 0.8^{c}
n6/n3	0.2 ± 0.0^{a}	0.3 ± 0.0^{b}	0.3 ± 0.0^{b}	0.4 ± 0.0^{c}
n3/n6	4.5 ± 0.1^{a}	3.9 ± 0.1^{b}	3.3 ± 0.2^{b}	2.6 ± 0.1^{c}
n9°	29.8 ± 0.4^{a}	30.1 ± 0.4^{a}	26.2 ± 0.4^{b}	24 ± 0.4^{c}

$^{+}$includes also 10:0, 13:0, 15:0, 17:0, 20:0, 21:0, 22:0, 23:0
*includes also 14:1n5, 15:1n5, 17:1n7, 24:1n9
$^{\#}$includes also 16:2n7, 18:3n6, 20:2n6, 20:3n6

^includes also 18:3n6, 20:2n6, 20:3n6
°includes also 24:1n9

3.5. Histology

No differences in the intestinal mucosa of all experimental groups were observed (Fig. 2) and no severe histological alterations were observed in all experimental groups in terms of inflammatory influx. The morphometric analysis of intestine folds evidenced a statistically significant reduction ($P < 0.05$) of intestinal folds length in HI50 (297.2 ± 43.0 μm) and HI75 (299.2 ± 47.4 μm) samples with respect to HI0 (374.2 ± 35.1 μm) and HI25 (347.6 ± 58.0 μm) samples. However, no evident differences were detected as regards the integrity of intestinal folds.

Concerning the histological analysis of the liver, no significant differences in lipid accumulation were detected in all the analyzed samples (Fig. 3). Regarding PAS stained sections, no differences in hepatocyte glycogen content were observed among the different experimental groups (Fig. 4).

3.6. FTIRM

Liver sections of clownfish fed the different experimental diets (HI0, HI25, HI50 and HI75) for 106 days (T1) were analyzed by FTIRM. In Fig. 5, the average absorbance spectra of all experimental groups were reported. Different spectral profiles were detected in HI0, HI25, HI50 and HI75 liver samples, mainly in the lipid region (3000-2824 cm^{-1}), in the band at ~1740 cm^{-1} attributable to the CO moiety of fatty acids, in the Amide I and II bands of proteins (~1660 and ~1550 cm, respectively), and in the carbohydrate region (1074-963 cm^{-1}).

The topographical distribution of lipids, fatty acids, proteins, and glycogen in the liver sections from the different experimental groups, was reported in Fig. 6 (for more details

see Material and methods section). Lower amounts of lipids and proteins, together with a higher amount of glycogen, were detected in HI50 and HI75 samples with respect to HI0 and HI25 samples. In HI75 samples, an increase of fatty acids was also observed with respect to all the other experimental groups.

To better analyze the effects of the different dietary treatments on the biochemical composition of clownfish liver, a semiquantitative analysis was performed on the band area ratios reported in Fig. 7. Regarding the relative amount of lipids (LIP/TBM; Fig. 7a), a statistically significant decrease ($P < 0.05$) was observed in HI50 and HI75 samples with respect to HI0 and HI25 samples. Conversely, fatty acids (FA/TBM; Fig. 7b) and saturated lipid alkyl chains (CH_2/TBM [Fig. 7c] and CH_2/LIP [Fig. 7d]) significantly increased ($P < 0.05$) with the increase of BSF inclusion. As regards proteins levels (PRT/TBM; Fig. 7e), a statistically significant decrease ($P < 0.05$) in HI50 and HI75 samples was detected with respect to HI0 and HI25 samples. Finally, as concerns glycogen levels (Fig. 7f, g and h), HI50 and HI75 samples showed a significant ($P < 0.05$) increase (GLY/TBM) with respect to HI0 and HI25 samples.

3.7. Real-Time PCR results

Real-time PCR analysis were performed on genes involved in fish growth (*igf1*, *igf2* and *mstn*), lipid metabolism (*pparα* and *pparβ*) and stress response (*gr* and *hsp70*). All biomarkers analyzed did not show significant differences ($P > 0.05$) among experimental groups (Fig. 8 a, b, c, d, e, f, and g) supporting the biometric data.

4. Discussion

As the marine ornamental aquaculture industry continues to grow and expand, it is essential to promote research to mitigate the current lack of information related to ornamental fish production (Olivotto et al., 2011; Calado et al., 2017).

In line with finfish aquaculture, one of the main marine ornamental aquaculture goals is to replace the unsustainable dietary FM and fish oil with more sustainable feed ingredients (Chong et al., 2003; Gasco et al., 2018a). However, new alternatives must ensure sustainability, economic feasibility and a proper fish growth and health by providing adequate levels of nutrients (Francis et al., 2001; Henry et al., 2015; Gasco et al., 2018a). Insects can exemplify good candidates for a more sustainable aquaculture, representing a valid alternative to FM and fish oil (Belghit et al., 2018; Bruni et al., 2018; Dumas et al., 2018; Henry et al., 2018; Gasco et al., 2018a; Vargas et al., 2018); however, to the best of our knowledge, no data are so far available for marine ornamental species.

Many studies on the inclusion of insects in aquafeed formulation have been carried out in the last years for finfish production (Kroeckel et al., 2012; Roncarati et al., 2015; Lock et al., 2016; Magalhães et al., 2017; Renna et al., 2017; Belghit et al., 2018; Gasco et al., 2018a; Henry et al., 2018; Vargas et al., 2018); however, results are still controversial and further research is necessary.

The present study, investigated for the first time in juvenile clownfish, the inclusion of different levels of BSF meal in their diets. In order to gain a deeper knowledge about the effects of the inclusion of BSF meal in aquafeeds, a comprehensive multidisciplinary approach was applied and results obtained may be useful to improve the understanding of the mechanisms involved in marine fish nutrition.

Generally, all the tested experimental diets did not show negative effects on clownfish growth and survival. No significant differences were observed in terms of SGR among

the experimental groups and these results were fully supported by the Real-Time PCR analyses on genes involved in fish growth such as *igf1*, *igf2* and *mstn*.

These findings were in line with those reported for other fish species such as Atlantic salmon (*Salmo salar*) (Lock et al., 2016), European seabass (*Dicentrarchus labrax*) (Magalhães et al., 2017), rainbow trout (*Oncorhynchus mykiss*) (Renna et al., 2017), yellow catfish (*Pelteobagrus fulvidraco*) (Xiao et al., 2018) and zebrafish (*Danio rerio*) (Vargas et al., 2018). However, in other fish species, such as turbot (*Psetta maxima*) and gilthead sea bream (*Sparus aurata*), the inclusion of high levels of insect meal in their diets resulted in lower growth and survival (Kroeckel et al., 2012; Gasco et al., 2016; Piccolo et al., 2017).

One of the well-known problems related to the inclusion of insect meal in aquafeeds is the presence of chitin which may induce a general reduction of the diet digestibility (Henry et al., 2015; Renna et al., 2017). The effects of the chitin in fish feeds is still not fully understood, and results are controversial. Moderate inclusion of chitin resulted in increased fish immune response (Esteban et al., 2000; Gopalakannan and Arul, 2006; Harikrishnan et al., 2012; Ringø et al., 2012; Zhang et al., 2012; Gasco et al., 2018b) and positive microbiota modulation (Askarian et al., 2012; Zhou et al., 2013; Bruni et al., 2018; Gasco et al., 2018b; Henry et al., 2018) but negative effects have also been reported because of possible intestinal inflammation and a reduced nutrient digestibility and assimilation (Kroeckel et al., 2012; Magalhães et al., 2017; Su et al., 2017; Xiao et al., 2018). For example, the inclusion of high percentages of BSF meal in rainbow trout (*Oncorhynchus mykiss*) and carp (*Cyprinus carpio*) diets induced intestine morphology changes and consequent effects on growth (Li et al., 2017; Dumas et al., 2018).

However, in the present study, no signs of severe intestinal inflammation were observed by the histological analyses in all the analysed samples, except for a reduction in intestinal

fold length that was exclusively observed in fish fed 50 and 75% BSF meal substitution diets. At this regard, shortened intestinal folds have previously been associated to impaired nutrient absorption and growth reduction (Moldal et al., 2014); however, in the present study this was not observed (see biometry section) and the molecular markers involved in stress response (*gr* and *hsp70*), that can be useful to precociously detect stress/inflammation physiological responses, did not show any significant difference among groups, suggesting a general fish welfare.

A possible explanation of the absence of intestine inflammation may be related to insect fatty acid composition. Insects are known to be rich in SFA (especially lauric acid [C12]), that have been reported to improve gut health because of their intestinal anti-inflammatory, antibacterial (Barroso et al., 2014; Paul et al., 2017; Feng et al., 2018; Gasco et al., 2018b; Henry et al., 2018; Vargas et al., 2018) and antiviral activity (Ponnuvel et al., 2003; Dayrit, 2015; Liland et al., 2017; Gasco et al., 2018b). In addition, medium chain fatty acids, mostly C6–C12, are considered physiologically active compounds which appear to be efficiently absorbed, digested and β-oxidised (as reviewed by Dayrit, 2015).

In conformity with data related to other fish species (Belforti et al., 2015; Gasco et al., 2016; Borgogno et al., 2017; Devic et al., 2017; Renna et al., 2017; Vargas et al., 2018; Zarantoniello et al., 2018; Zhou et al., 2018), while the total lipid content of clownfish was similar among groups, their FA composition reflected the FA composition of the diets. In particular, high dietary BSF inclusion levels caused an increase in SFA and a reduction in PUFA in the fish. The regulation of lipid homeostasis in fish is a complex balance between lipid uptake, transport, storage, energy utilization, and biosynthesis. Such processes are controlled independently but also in conjunction with others (Tocher, 2003; Leaver et al., 2008).

At this regard, the liver plays a key role in fish lipid metabolism and its morphological structure, macromolecular composition and gene expression are deeply influenced by the diet. In the present study, as already observed in other fish species fed on insect based diets (Davis et al., 1999; Lock et al., 2016; Rapatsa and Moyo, 2017), no differences in the amount of hepatic lipid accumulation was observed among the experimental groups. In accord to this finding, *PPARs*, which are known to respond to lipids and elicit transcriptional changes on genes involved in lipid metabolism in fish and mammals (Ayisi et al., 2018), did not show differences in their gene expression.

The liver also plays a key role in the control of endogenous carbohydrate storage and glucose mobilization in fish (Metón et al., 2003; Sangiao-Alvarellos et al., 2005; Pérez-Jiménez et al., 2007; Viegas et al., 2012). Carbohydrates and lipids are two important non-protein energy sources for fish (Bing-Ke et al., 2017), and usually have a close relationship with each other in glycolipid metabolism (Zhou et al., 2016). In the same liver samples, histological PAS technique did not reveal diet-dependent modifications in glycogen accumulation.

Even if histology is one of the gold standards for morphological analysis because it allows to study large biological sections and to examine the internal architecture of various tissues and cells, this technique does not always provide sufficient information about the biochemical composition of tissue samples. At this regard, in the present study, the effects of different diets on the composition of clownfish liver was also determined by Fourier Transform Infrared Microspectroscopy (FT-IRM). In detail, FT-IRM analyses performed on clownfish liver samples evidenced, in accord to previous studies (Gioacchini et al., 2014; Carnevali et al., 2017; Forner-Piquer et al., 2017; Randazzo et al., 2018) that the diet was able to modulate the biochemical composition of liver. In particular, an increase of BSF meal in the diet resulted in an increase of saturated lipid alkyl chains (CH2/TBM

and CH2/LIP) in the hepatic lipids and a higher glycogen deposition (GLY/TBM, GLY/PRT and GLY/LIP).

These results suggested that 1) clownfish, as marine fish species, are unable to convert shorter-chain FA precursors (particularly abundant in insects) in highly unsaturated FAs; 2) clownfish fed diets with high BSF inclusion levels accumulated hepatic glycogen.

While it is well known that marine fish species generally lack the ability to convert shorter-chain FA precursors in long-chain polyunsaturated fatty acids (LC-PUFAs) due to deficiencies in specific desaturase or elongase enzymes (Sargent et al., 1999), results about glycogen accumulation were not obvious.

It should be pointed out that the carbohydrate utilization and metabolism of fish is quite complex, and has been reported to be affected by a large number of factors including feeding habits, species, genotypes, digestive system functions, growth and/or developmental stages, dietary compositions, feed manufacturing, feeding regimes and water temperature (Hung and Storebakken, 1994; Enes et al., 2009; Polakof et al., 2012; Bing-Ke et al., 2017).

5. Conclusions

The present study tested for the first time partial dietary inclusions of *Hermetia illucens* meal in clownfish diets and the physiological responses of the fish. Generally, results are promising since no negative effects on growth, stress response and survival were detected. However, the dietary inclusion of BSF meal affected the fatty acid composition of the fish. Further studies are needed to better understand the physiological responses of clownfish fish over a longer period of time to evaluate possible effects on the different stages of their life cycle, with particular emphasis on the reproductive stage and the possible effects on gamete quality.

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Figures

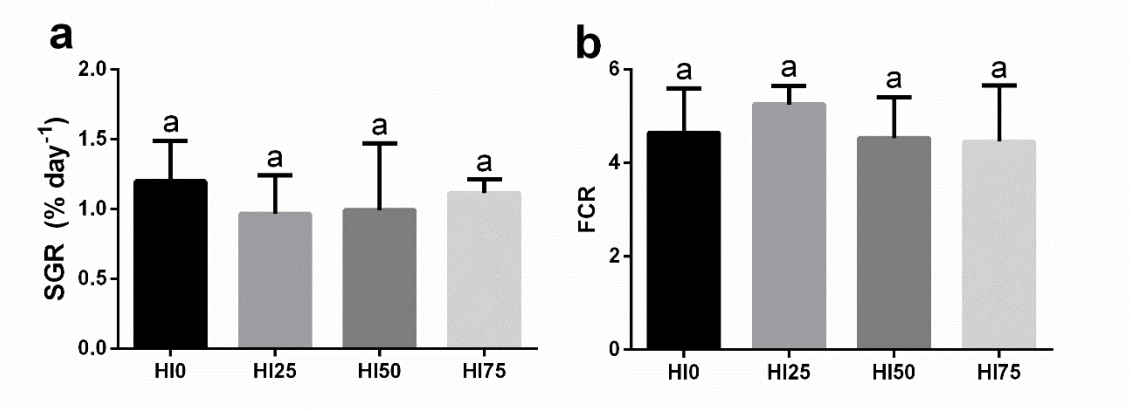

Fig. 1. Specific growth rate (SGR % increase in wet weight day^{-1})(a) and feed conversion ratio (FCR) (b) in *A. ocellaris* juveniles fed diets where FM was substituted by 0 % (HI0), 25 % (HI25), 50 % (HI50) and 75 % (HI75) BSF meal. No significant differences among groups were detected (P > 0.05).

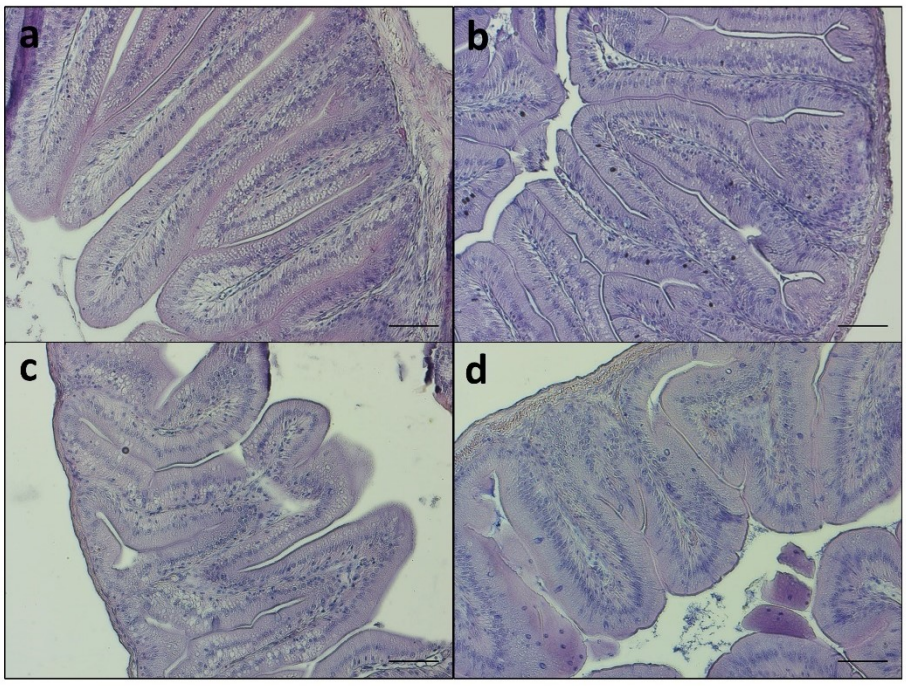

Fig. 2. Intestine histology (H&E) of *A. ocellaris* juveniles fed diets where FM was substituted by 0 % (a), 25 % (b), 50 % (c) and 75 % (d) BSF meal, sampled at the end of the experiment (106 days). Scale bars: 50 µm.

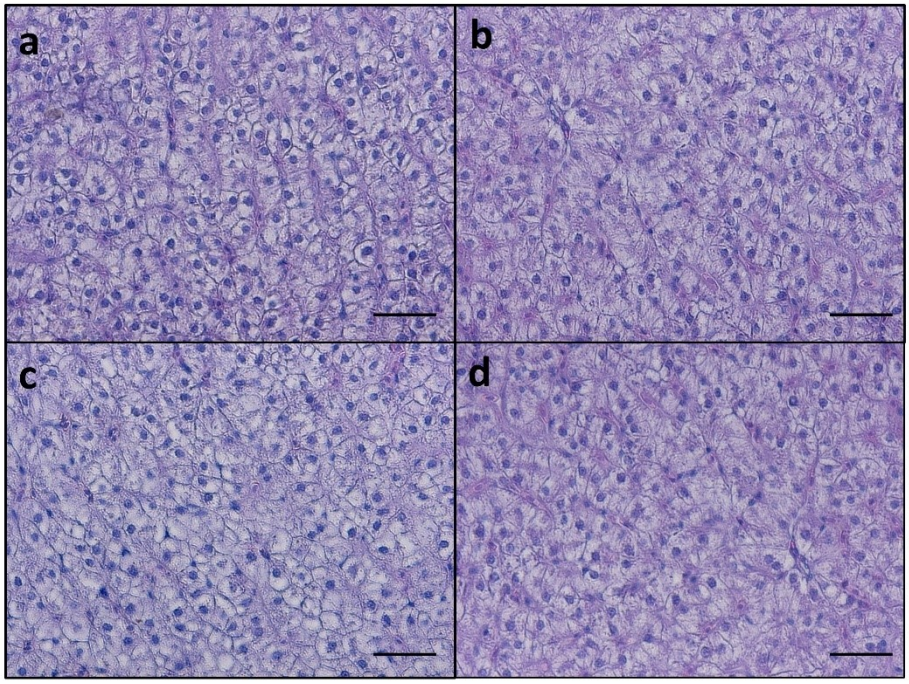

Fig. 3. Liver histology (H&E) of *A. ocellaris* juveniles fed diets where FM was substituted by 0 % (a), 25 % (b), 50 % (c) and 75 % (d) BSF meal, sampled at the end of the experiment (106 days). Scale bars: 20 µm.

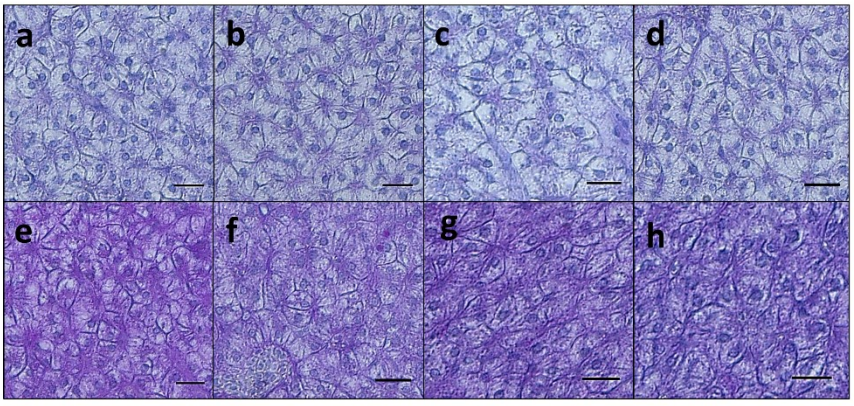

Fig. 4. Livers of *A. ocellaris* stained with periodic acid-Schiff (PAS), digested (a, b, c, d) and not digested (e, f, g, h) with α-amylase. Fish were fed diets where FM was substituted by 0 % (a, e), 25 % (b, f), 50 % (c, g) and 75 % (d, h) BSF meal, and sampled at the end of the experiment (106 days). Scale bars: 10 µm.

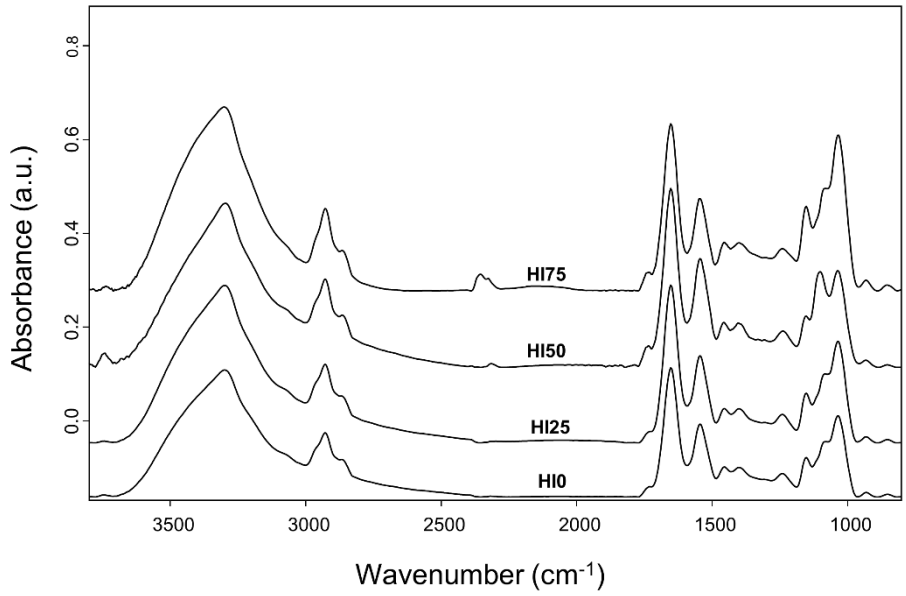

Fig. 5. Average absorbance spectra of liver sections of *A. ocellaris* juveniles fed diets where FM was substituted by 0 % (HI0), 25 % (HI25), 50 % (HI50) and 75 % (HI75) BSF meal, sampled at the end of the experiment (106 days). Spectra were reported in absorbance in the 3800-950 cm^{-1} spectral range.

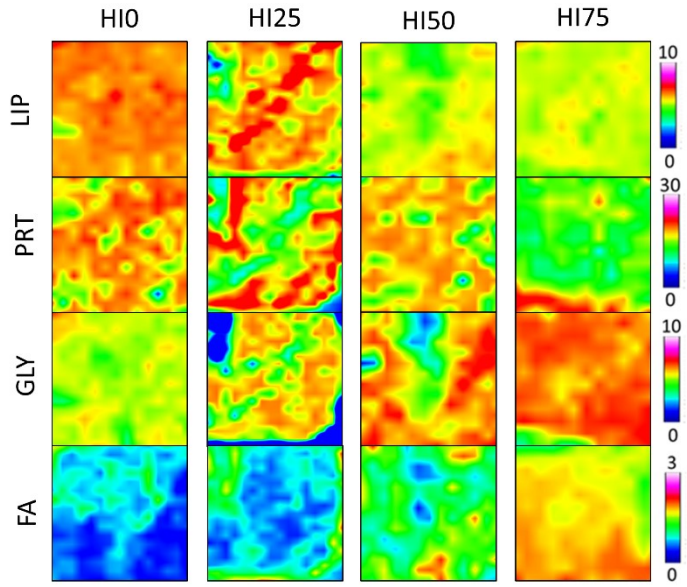

Fig. 6. Representative false color images of liver sections from HI0, HI25, HI50 and HI75 experimental groups, showing the topographical distribution of lipids (LIP), fatty acids (FA), proteins (PRT) and glycogen (GLY). White color indicates higher absorbance values, whilst blue color indicates lower ones.

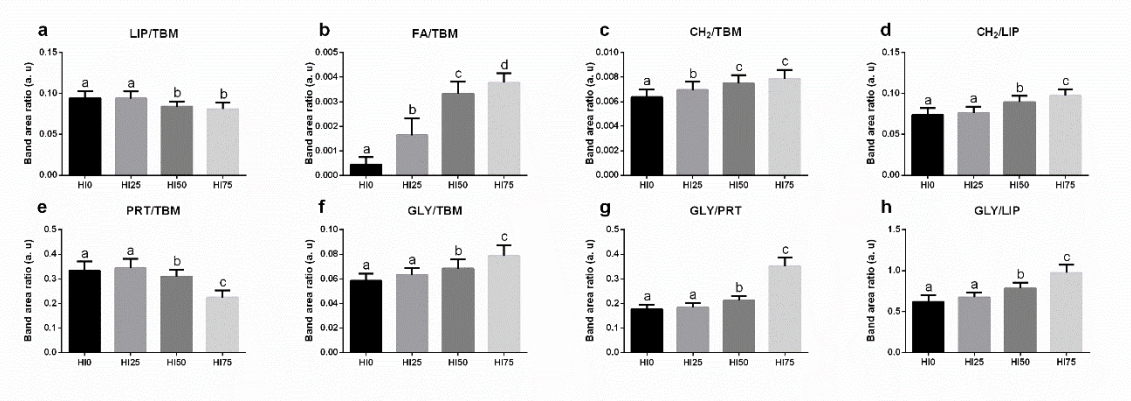

Fig. 7. Statistical analysis of the following band area ratios with biological relevance: LIP/TBM (a), FA/TBM (b), CH_2/TBM (c), CH_2/LIP (d), PRT/TBM (e), GLY/TBM (f), GLY/PRT (g), and GLY/LIP (h). Fish were fed diets where FM was substituted by 0 % (HI0), 25 % (HI25), 50 % (HI50) and 75 % (HI75) BSF meal, sampled at the end of the experiment (106 days).

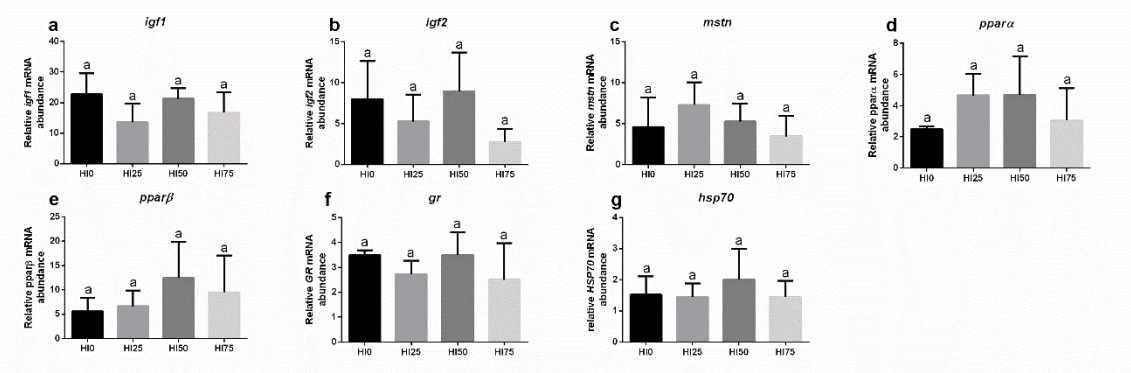

Fig. 8. Relative mRNA abundance of genes involved in fish growth (*igf1*[a], *igf2* [b] and *mstn* [c]), lipid metabolism (*pparα* [d] and *pparβ* [e]), and stress response (*gr* [f] and *hsp70* [g]), analyzed in liver samples of *A. ocellaris* juveniles fed diets where FM was substituted by 0 % (HI0), 25 % (HI25), 50 % (HI50) and 75 % (HI75) BSF meal,

sampled at the end of the experiment (106 days). No significant differences were observed among experimental groups ($P > 0.05$).

Chapter 4

Chapter 4 - Exploring seaweeds as feed additives and medaka as a model for feed-induced intestinal inflammation

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Abstract

Intestinal inflammation due to the use of alternative feed ingredients to fish meal and fish oil in aquafeeds, such as soybean meal, is a major challenge in aquaculture. In the present study, medaka (*Oryzias latipes*), an emerging fish model, was evaluated as a potential model for feed-induced intestinal inflammation. To that end, 80 juvenile medaka were fed for 25 days with challenge diets including soybean meal (35%) and pure saponins (0.5%), as well as cholesterol (3%) (Krogdahl et al. 2010). Moreover, a possible anti-inflammatory role of two seaweeds, the red seaweed *Palmaria palmata* and the brown seaweed *Saccharina latissima,* at 5% dietary inclusion level was evaluated. The challenge diets did not induce any sign of inflammation in either the proximal or the distal intestine, as revealed by histology and gene expression analysis of stress, immune response, enteritis, tissue recovering and cholesterol metabolism markers. Seaweeds did not induce any effect on fish growth and gut health parameters. To our knowledge, this was the first study evaluating a possible feed-induced intestinal inflammation model in the medaka genus, and our results indicated that *O. latipes* can tolerate the tested levels of dietary soybean meal, saponins and cholesterol without a typical gut inflammatory response being triggered.

Key words: Soybean, inflammation, seaweeds, feed additives, intestine, fish model, aquaculture.

Introduction

Aquaculture is presently the fastest growing food sector in the world and is hoped to meet the rising food demand (FAO 2018). Fishmeal has long been considered a cost-effective, high quality protein for aquafeed manufacture but it can no longer sustain the expansion of aquaculture in both environmental and economic terms (Hargreaves et al. 2019). Among available alternative feed ingredients, soybean meal (SBM) is a major substitute due to its high abundance, low price and high nutrition value (Bandara 2018). Unfortunately, SBM, like most plant ingredients, contains a wide variety of anti-nutritional substances, such as saponins and lectins, that negatively impact the feed quality and the gut health (Krogdahl and Bakke 2015).

The most common gastrointestinal problem associated with the use of SBM in fish feed is intestinal inflammation in the distal intestine characterized by a shortening of the mucosal folds, widening of the lamina propria and sub-epithelial mucosa associated with a strong infiltration of various inflammatory cells. This pathology has been observed in several fish species that include Atlantic salmon (*Salmo salar*) (Krogdahl et al. 2003), common carp (*Cyprinus carpio*) (Urán et al. 2008), rainbow trout (*Oncorhynchus mykiss*) (Yamamoto et al. 2008), juvenile turbot (*Scophthalmus maximus*) (Gu et al. 2016), Japanese flounder (*Paralichthys olivaceus*) (Chen et al. 2011), as well as zebrafish (*Danio rerio*) larvae (Coronado et al. 2019). In Atlantic salmon, SBM dietary inclusion levels as low as 10% induced a mild reduction of microvilli size as well as a reduction in pinocytotic vesicles in the Di (Urán et al. 2009). Further, 10% SBM levels gave a reduction in mucosal enzyme activities in the distal intestine, which coincided with reductions in apparent digestibility of lipid, energy and crude protein and growth rate (Krogdahl et al. 2003). A recent study, using the powerful zebrafish model, was able to monitor in vivo the participation of innate and adaptive cells in just hatched zebrafish, highlighting the advantages of fish models in the understanding of biological processes associated with intestinal inflammatory diseases (Progatzky et al. 2014; Coronado et al. 2019).

The Japanese medaka, Oryzias latipes, is an emerging fish model complementary to zebrafish (Kinoshita et al. 2012a). Advanced tools are widely applied for medaka studies thanks to an extensive amount of information made available by genome and transcriptome sequencing (Kinoshita et al. 2012d). In addition, all known zebrafish techniques also apply to medaka (Kinoshita et al. 2012c). Medaka is a cheap and easy-to-keep aquarium fish, with a high fecundity and short generation time, thus a potent model

for nutritional studies (Kinoshita et al. 2012c). In addition, due to their high salinity tolerance it can be a model not only for freshwater fish, but also for marine and brackish water fish, including the commercially important Atlantic salmon.

To the author's knowledge, there are no published studies assessing diet-induced intestinal inflammation in medaka. Most research efforts involving stress and immune response in medaka have been focused on effects triggered by other stressors. These include pathogens (Huang et al. 2012), cardiac damage (Lai et al. 2019), medications (Kuwashiro et al. 2011) and wound healing (Takeyama et al. 2016).

Seaweeds represent an interesting fish meal replacement and feed additive with potential anti-inflammatory properties (Rajauria 2015). Besides their high nutrition value (Peng et al. 2015), they offer a rich source of bioactive compounds, including novel dietary fibres, polyphenols, fatty acids, and carotenoids (Angell et al. 2016). Studies in different fish species associated the seaweed administration to positive health effects such as improved immune response and disease resistance (Cruz-Suárez et al. 2009; Sotoudeh and Jafari 2017; Telles et al. 2018).

The aim of the present study was to explore medaka as a model for feed-induced intestinal inflammation. To that end, diets were formulated including high levels of soybean meal (35%), purified soy saponins (0.5%), as well as cholesterol (3%) as an additional stressor based on previous experiment in rats and zebrafish (Progatzky et al. 2014). Further, two macroalgae species were evaluated for their possible immune-modulatory role. Seaweeds are innovative, environmentally friendly feed additives (Rajauria 2015) that have been reported to have beneficial health effects in both humans and animals (ref). Gut health was assessed through histological observations of the proximal and distal intestine and through the evaluation of expression levels of molecular markers involved in stress and immune responses, tissue recovering, enteritis and cholesterol metabolism.

Materials and methods

Macroalgae source

The red seaweed *Palmaria palmata* was harvested by the Norwegian Institute of Bioeconomy Research (NIBIO) in May 2015 in the Bodø-area of the Skjerstad Fjord, Norway. The brown seaweed *Saccharina latissima* was cultivated by Stiftelsen for industriell og teknisk forskning (SINTEF, Trondheim, Norway) at the Seaweed Energy Solutions AS cultivation field near the island Frøya in Norway, and harvested in June 2015. The whole macroalgae products, once collected, were kept frozen (-20 °C), freeze dried and minced for further feed processing.

Diets

Three formulated challenge diets (Group A, Group B and Group C) and a commercial diet (Control) were used in the present study (Table 1). Among these, three isonitrogenous, isolipidic and isoenergetic diets were formulated and manufactured by Sparos Lda. (Olhão, Portugal). A basal diet (Group A) included, as a dietary challenge, high levels of soybean meal (35%), purified soy saponin (0.5%) and cholesterol (3%) to induce an intestinal inflammatory response. To evaluate the possible protective effects of seaweeds, the basal diet (Group A) was supplemented with 5% of *S. latissima* (Group B) or 5% *P. palmata* (Group C) whole macroalgae product, replacing wheat meal, respectively. All powder ingredients were initially mixed and ground in a micropulverizer hammer mill (SH1, Hosokawa-Alpine, Germany). Subsequently, the oils were added to the powder mixture, which were moisturised with approximately 25% water and agglomerated by a low-shear and low-temperature extrusion process (ITALPLAST, Italy). The resulting pellets were dried in a convection oven for 4 h at 40 ºC (OP 750-UF, LTE Scientifics, United Kingdom), and afterwards crumbled and sieved (400 µm).

As a control (Control) to the formulated diets, a commercial feed (Gemma Wean®, Skretting, Norway) was included in the experimental design (Table 1). No information regarding composition of the feed was available due to commercial reasons.

Table 1. Ingredients and proximal composition of the experimental diets.

	Control	Group A	Group B	Group C
Ingredients (% of inclusion)				
MicroNorse	-	25	25	25
CPSP 90	-	5	5	5
Fish gelatine	-	2.5	2.5	2.5
Soybean meal 48	-	35	35	35
Wheat meal	-	21.3	16.3	16.3
Fish oil - Sopropeche	-	1.2	1.2	1.2
Palm oil	-	4.2	4.2	4.2
Vit & Min Premix INVIVO 1%	-	2	2	2
Antioxidant powder (Verdilox)	-	0.3	0.3	0.3
Saponins	-	0.5	0.5	0.5
Cholesterol SF	-	3	3	3
S. latissima	-	-	5	-
P. palmata	-	-	-	5
Proximate composition				
Crude protein (% of inclusion)	-	44.1	43.9	43.9
Crude fat (% of inclusion)	-	12.2	12.2	12.2
Fiber (% of inclusion)	-	1.7	4.1	4.1
Ash (% of inclusion)	-	8.7	10.7	10.7
Gross Energy (MJ/kg feed)	-	21.6	21.2	21.2

Fish husbandry

Japanese medaka (*Oryzias latipes*) were spawned and maintained for three months at the medaka fish facilities of the Department of Basic Sciences and Aquatic Medicine, NMBU, Oslo, Norway (https://www.nmbu.no/) in a re-circulating system under the following conditions: pH 7; water temperature 28 ± 1 °C; NO2 and NH3 < 0.01 mg L-1; N03 < 10 mg L-1; and photoperiod 12 L:12 D. System water was treated with mechanical, biological and UV filtration. The fish were fed three times per day with a combination of dry feed (Gemma Wean®, Skretting, Norway) and live brine shrimp nauplii larvae (*Artemia salina*).

Experimental design

Eighty fish were randomly divided in eight tanks of 10 L (10 fish per tank) and fed twice a day until apparent satiation for 25 days according to the four experimental diets (two tanks per diet): Control: Fish fed a commercial dry feed (Gemma Wean®, Skretting, Norway). Group A: Fish fed the formulated challenge diet (Group A). Group B: Fish fed the formulated challenge diet that included 5 % of *S. latissima (Group B)*. Group C: Fish fed the formulated challenge diet that included 5 % of *P. palmata (Group C)*.

Fish were fasted 20 hours prior to sampling to ensure intestines were free of intestinal content. The intestine was separated into proximal (Pi) and distal (Di) regions according to a previous study that demonstrated that the first four sections of the medaka intestine, when divided in six equal sections, displayed transcriptional homology to the small intestine (Aghaallaei et al. 2016), whereas the last fifth and sixth fractions to the human large intestine. Fish were euthanized with ice water, and tissue samples properly stored for further analysis.

Biometry

At the end of the experiment, the wet weight of all sampled fish was measured with an OHAUS Explorer analytical balance (Pine Brook, New Jersey, USA; precision 0.1 mg), and sex was determined according to the size and shape of the anal fin (Kinoshita et al. 2012b).

Histology

Ten fish randomly collected at the end of the experiment from the two tanks (five per tank), belonging to each dietary treatment (ten per dietary treatment), were dissected to remove the intestines. The samples were fixed by immersion in formalin and stored at 4 °C for 24 h. The samples were then washed and preserved in ethanol (70%). Samples were routinely dehydrated in ethanol, equilibrated in xylene, and embedded in paraffin according to standard histological techniques. The paraffin blocks were placed on a cooling block before cut by an automatic microtome (HM 355S, Thermo Scientific™, US) to produce sections of 4 μm thickness and stained with hematoxylin and eosin (H&E).

The slides were blindly examined with a light microscope (Axio Scope.A1, Zeiss, Germany) equipped with a camera (AxioCam ICc 3, Zeiss) paying attention to typical

inflammatory morphological changes observed in other fish species fed SBM diets, including shortening and fusion of mucosal folds, cellular infiltration within the lamina propria and submucosa, enterocyte vacuolization and nucleus position disparity.

RNA extraction and cDNA synthesis

Total RNA extraction from Pi and Di samples obtained from ten fish per experimental group, randomly collected from the two tanks per diet at the end of the experiment, was optimized using RNAzol® RT reagent (Sigma-Aldrich®, R4533) following the manufacturer's instructions. RNA purity and concentration were measured using Epoch Microplate Spectrophotometer (BioTeK Instruments, Winooski, VT, USA) and the integrity was verified by the 2100 Bioanalyzer with the 6000 Nano LabChip kit (Agilent Technologies, Palo Alto, CA, USA). The mean A260/A280 ratio was 2.05 (Standard Deviation=0.2) and the average RIN (RNA integrity number) value was 8.6 (S.D.=0.51). First-strand complementary DNA (cDNA) synthesis was performed using 1.0 µg total RNA from all samples using a Superscript™ IV VILO™ cDNA synthesis kit (catalog no., 11756050) (Invitrogen, Carlsbad, CA, USA). A negative control was included by omitting RNA and the obtained cDNA was diluted 1:10 before further use.

Real-time quantitative polymerase chain reaction (qPCR)

The real-time qPCR primers were obtained from the literature or designed using Primer-BLAST (http://www.ncbi.nlm.nih.gov/tools/primer-blast/). Primer specificity for reference and target genes were evaluated *in silico* by the tool Primer-BLAST (Ye et al. 2012) available at http://www.ncbi.nlm.nih.gov/tools/primer-blast/. Specificity was also verified by a melting curve after each PCR assay and subsequent agarose gel electrophoresis to confirm the amplification of a single product with the expected molecular size and absence of primer-dimers. Primer efficiency was determined using 2-fold serial dilutions of randomly pooled cDNA. The PCR assays were performed using the LightCycler 96 (Roche Applied Science, Basel, Switzerland) and a 10-µl reaction volume was used, which contained 2 µL of PCR-grade water, 2 µL diluted cDNA template, 5 µL LightCycler 480 SYBR Green I Master (Roche Applied Science) and 0.5 µL (10 µM) of each forward and reverse primer. Samples were run in duplicates in addition to a no-template control for each gene. A three-step PCR programme was applied incorporating an enzyme activation step at 95 °C (5 min) and 40 cycles of 95 °C (10 s), 60 °C (10 s) and 72 °C (15 s). The plate pipetting was done using the Biomek® 4000

automation workstation (Beckman Coulter, Fullerton, CA, USA). Quantification cycle (Cq) values were determined using the second derivative method. The specificity of PCR amplification was confirmed by evaluating the melting curve of PCR products and the band pattern on the agarose gel after electrophoresis. The expression of target genes in both Pi and Di samples was normalized to the geometric mean of glyceraldehyde-3-phosphate dehydrogenase (*gapdh*) and ribosomal protein L7 (*rpl7*), after evaluation of their stability across and within treatments (Kortner et al. 2011). The mean normalized expression of the target genes was calculated from raw Cq values (Muller et al. 2002). The genes profiled and the primers used for the qPCR assays are given in Table 2.

Table 2. Primer sequences used in the present study.

Gene	Forward (5'- 3')	Reverse (5'- 3')	Acc number	Efficiency	Reference
il1b	CTGTTTCTGGAGGAGGTGG	AGAAGAGGAAGCGCACATT	XM_011478737.2	1.91%	(Singha et al. 2019)
il10	GACAATCTTGCCCAAAGCGG	CAGTCCTGAGCTGGTCGAAA	XM_004069264	1.98%	This study
tgfb2	GTTACTCCGACCTGAGGAAGATAG	TGACACCCAATCTTTAACGGTTTC	XM_004073149.3	1.91%	(Singha et al. 2019)
cat	TGCTAGCAGTTGATTGTCTGT	CACAGATCCACTGAAACAGGA	XM_004069460.2	1.96%	(Singha et al. 2019)
tnfa	AACCGAAGAGTCTGAGAGGG	AGCTGAAGAAGAGTACCGCT	XM_004074335.3	2.04%	(Singha et al. 2019)
fabp2	GCTGCTCACGACAACCTCAA	CCATGCACCCGATAGTTCAG	XM_004084292.3	1.96%	(Aghaallaei et al. 2016)
fabp6	CAAGTTCACCATTGGGAAGG	ATGAGCTTGCCTCCACTGAT	XM_023965490.1	1.93%	(Aghaallaei et al. 2016)
aqp8ab	GCTGCTAAGCCTCCCAGTAA	CCGACACACAACCAATGAAG	XM_023949431.1	2.1%	(Madsen et al. 2014)
ctsl1	TCCATGGGCAAACACTCGTA	AGTTGGGCTCCATGAACAGG	NM_001104686.1	1.91%	(Aghaallaei et al. 2016)
mmp9	AAGTGCGTCTTCCCTTTCGT	TGGTCGTAGTTGCTTGTGGT	NM_001104880.1	1.96%	This study
mmp13	GCACAAATAGCCCTGCGATG	TTTGGGGGTCATAGGGTTGC	NM_001104715.1	1.88%	This study
pcna	ATCTCCTGTGCCAAAGACGG	CAATGGTGACGGCCTCTTCT	XM_004072187.4	2.02%	This study
srebf2	AGAACACCGCTCTCCAGGT	GTTCCATGCTCGGTCTGACT	XM_011478517.3	1.97%	This study
cyp7A1	TGTCAGTAAAGCCAGGACCCATGT	TCCAGAGCCAAAGGGCATGTAGAA	XM_004078870.3	1.82%	(Howarth et al. 2010)
apoa1	GATCAAAGCCCTGGATCAGC	TGGCATCAGAAATGGTGGTG	XM_023961457.1	1.94%	(Aghaallaei et al. 2016)
gapdh	CCTCCATCTTTGATGCTGGT	ACGGTTGCTGTAGCCAAACT	XM_004077972.3	2%	(Burow et al. 2020)
rpl7	TGCTTTGGTGGAGAAAGCTC	TGGCAGGCTTGAAGTTCTTT	NM_001104870	1.95%	(Burow et al. 2020)

Statistics

Fish wet weight data was analysed by one-way ANOVA with diet as explanatory variable and by t-test with fish sex as explanatory variable. Real-time PCR data was analysed by one-way ANOVA, with diet as explanatory variable. Data was tested for normality using the Shapiro-Wilk test. Data that did not pass the test was then carefully inspected through an exploratory analysis, that included data transformation, which revealed that violation of normality was due to the high variability of the data rather than for their distribution nature. Homogeneity of variance was verified through residual plots according to Zuur et al. (2007). All ANOVA tests were followed by Tukey's post hoc test, when significant differences were detected. The statistical software package Prism5 (GraphPad Software) was used; significance was set at $p < 0.05$. All results are presented as mean ± SEM.

Results

Biometry

All the animals used for the present study survived until the end of the experiment. Considering the fish wet weight, both Sacc (0.27 ± 0.01g) and Palm (0.27 ± 0.01g) groups did not show any significant difference ($p > 0.05$) with respect to the control group (0.27 ± 0.01g) at the end of the experimental period, while Gemma group (0.33 ± 0.01g) exhibited a significantly higher ($p < 0.05$) wet weight with respect to all other experimental groups (Fig. 1a). Considering the wet weight of fish grouped by sex, females (0.29 ± 0.01 g) exhibited a slightly higher, but significant ($p < 0.05$), wet weight with respect to males (0.27 ± 0.00 g; Fig. 1b).

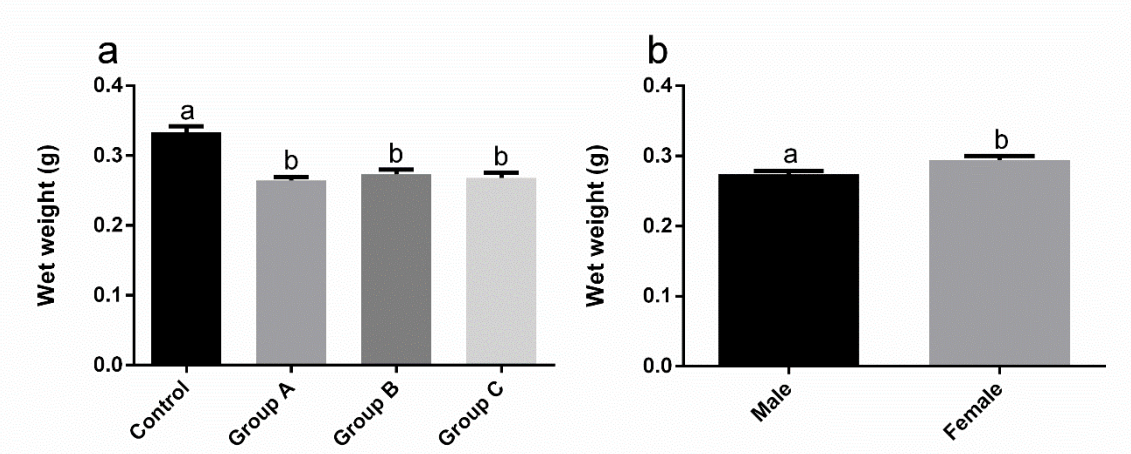

Fig. 1. Fish wet weight (g) in *O. latipes* juveniles fed a commercial diet (Control), a challenge diet (Group A) and challenge diets including 5% of *S. latissima* (Group B) and 5% of *P. palmata* (Group C), sampled at the end of the experiment (25 days). Different letters above each column indicate statistically significant differences among experimental groups ($p < 0.05$). Values are presented as mean ± SEM (n = 20 for Fig. 1a and n = 40 for Fig. 1b).

Histology

No appreciable differences among the Pi and Di intestinal mucosa of all experimental groups were detected after 25 days of dietary treatment (Fig. 2). No severe histological alterations were observed in all experimental groups in terms of inflammatory influx and alterations of intestinal folds.

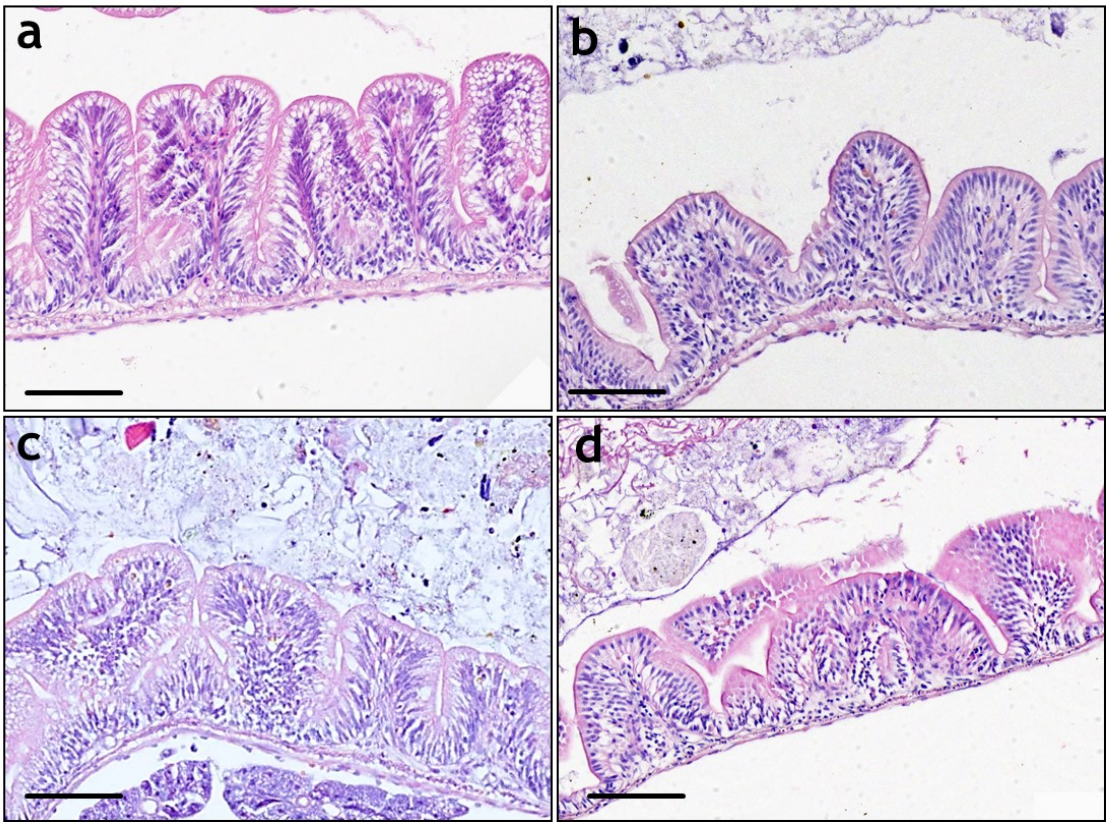

Fig. 2. Proximal intestine histology (H&E) of *O. latipes* juveniles fed a commercial diet (Control), a challenge diet (Group A) and challenge diets including 5% of *S. latissima* (Group B) and 5% of *P. palmata* (Group C), sampled at the end of the experiment (25 days). Scale bars: 50 µm.

Real-time PCR results

Real-time PCR analyses were performed on Pi and Di samples in order to test the expression of genes involved in fish stress response (catalase [*cat*] and tumor necrosis factor alpha [*tnfa*]), immune response, including pro-inflammatory (interleukin 1 beta [*il1b*] and transforming growth factor beta 2 [*tgfb2*]) and anti-inflammatory markers (interleukin 10 [*il10*]), tissue recovering (matrix metallopeptidase 9 [*mmp9*], matrix metallopeptidase 13 [*mmp13*] and proliferating cell nuclear antigen [*pcna*]), normal gut

function typically affected in SBM-induced enteritis (fatty acid binding protein 2 [*fabp2*], fatty acid binding protein 6 [*fabp6*], aquaporin 8ab [*aqp8ab*] and cathepsin L [*ctsl.1*]) and sterol metabolism (sterol regulatory element binding transcription factor 2 [*srebf2*], cytochrome P450 family 7 subfamily A member 1 [*cyp7a1*] and apolipoprotein A1 [*apoa1*]).

Stress, immune response and tissue recovering markers. As regards all the markes analyzed for stress (*cat* and *tnfa;* Fig. 3), immune response (*il1b*, *il10* and *tgfb2*; Fig. 4) and tissue recovering (*mmp9*, *mmp13* and *pcna*; Fig. 5), no significant differences ($p > 0.05$) were detected among the experimental groups in both Pi and Di samples.

Enteritis markers. Conversely, as regards enteritis markers, significant differences ($p < 0.05$) were detected in Pi samples. Specifically, regarding *fabp2* gene expression, all challege groups (Group A, B and C) showed a significantly lower ($p < 0.05$) gene expresion level with respect to Control (Fig. 6a). A similar result was observed for *fabp6* gene expression (Fig. 6c). For *aqp8ab* gene expression, an opposite trend was observed (Fig. 6e). Particularly, all challenge groups (Group A, B and C) showed a significantly higher ($p < 0.05$) gene expression level with respect to Control (Fig. 6e). As concerns *ctsl.1* gene expression, no significant differences ($p > 0.05$) were evident among the experimental groups (Fig. 6g). In Di samples (Fig. 6b, d, f and h), no significant differences were detected for all the markers analyzed.

Sterol metabolism. Considering sterol metabolism, significant differences ($p < 0.05$) were detected in Pi samples. In particular, regarding *srebf2* gene expression, all challenged groups (Group A, B and C) showed a lower gene expression with respect to the Control group, significantly ($p < 0.05$) only for Group B with respect to Control (Fig. 7a). As concerns *cyp7a1* gene expression, no signal was detected in all the analysed samples (Fig. 7b). Considering *apoa1* gene expression, no significant differences were observed among experimental groups (Fig. 7c). In Di samples, no significant differences were detected for all the markers analysed (Fig. 7d, e and f).

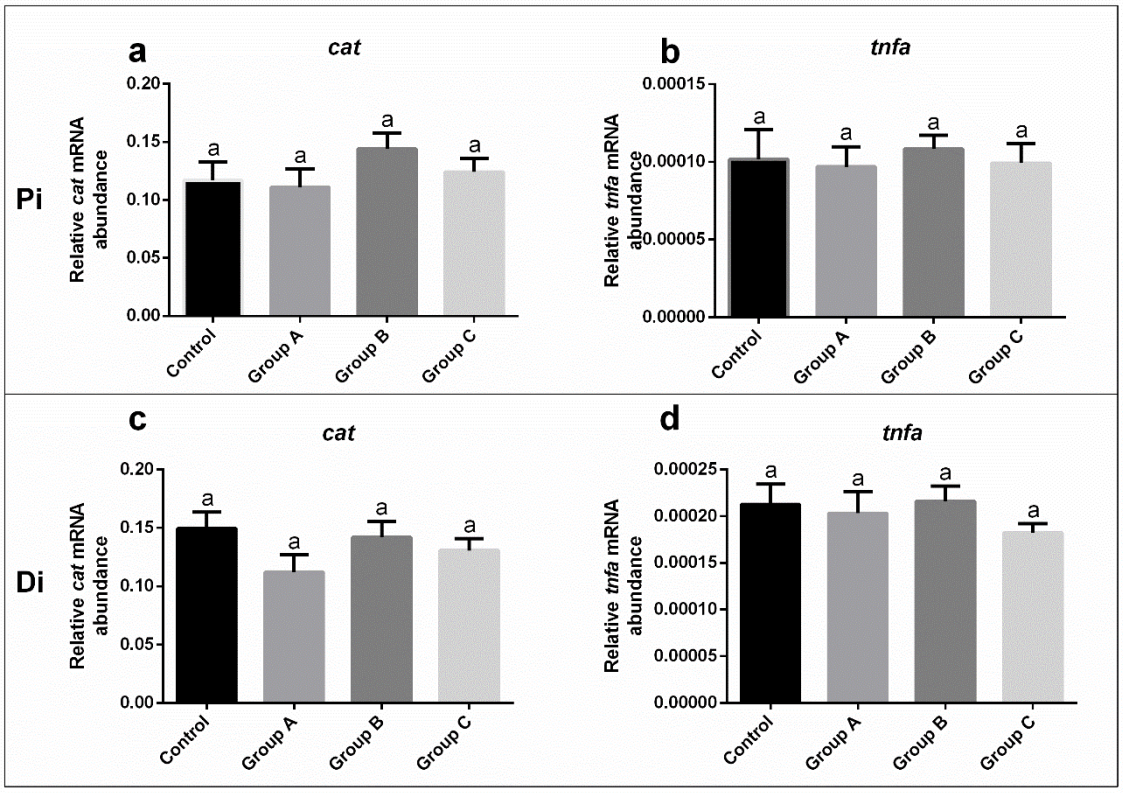

Fig. 3. Relative mRNA abundance of genes involved in stress response (*cat* and *tnfa*) analysed in *O. latipes* proximal (Pi) and distal intestine (Di), fed a commercial diet (Control), a challenge diet (Group A) and challenge diets including 5% of *S. latissima* (Group B) and 5% of *P. palmata* (Group C), sampled at the end of the experiment (25 days). Values are presented as mean ± SEM (n = 10). No significant differences were observed among experimental groups ($p > .05$), as indicated by the letter above each column.

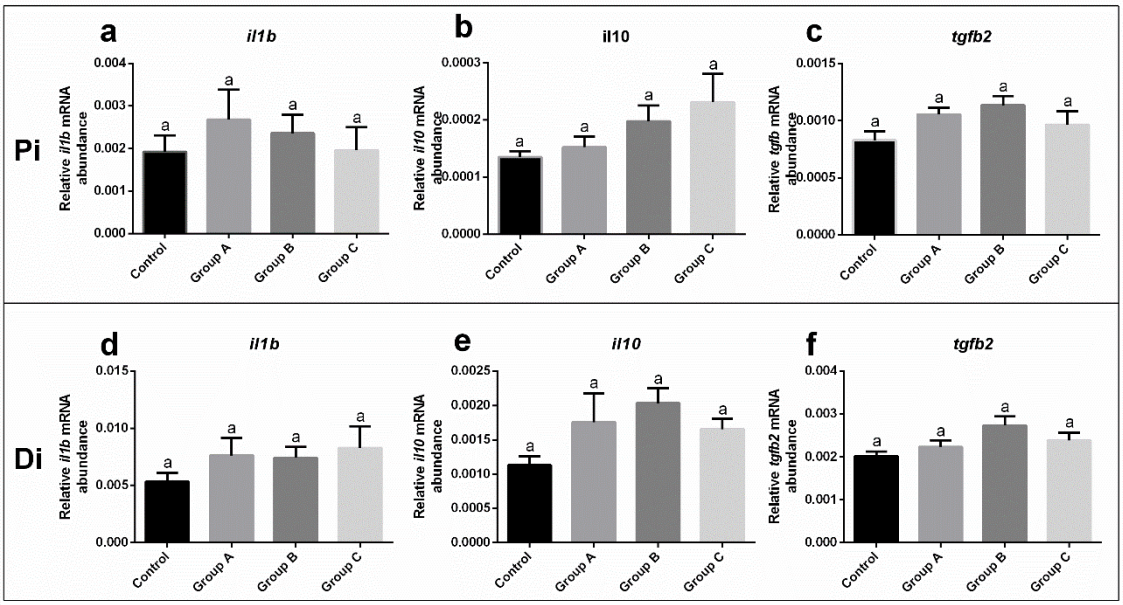

Fig. 4. Relative mRNA abundance of genes involved in immune response (*il1b*, *il10* and *tgfb2*) analysed in *O. latipes* proximal (Pi) and distal intestine (Di), fed a commercial diet (Control), a challenge diet (Group A) and challenge diets including 5% of *S. latissima* (Group B) and 5% of *P. palmata* (Group C), sampled at the end of the experiment (25 days). Values are presented as mean ± SEM (n = 10). No significant differences were observed among experimental groups ($p > .05$), as indicated by the letter above each column.

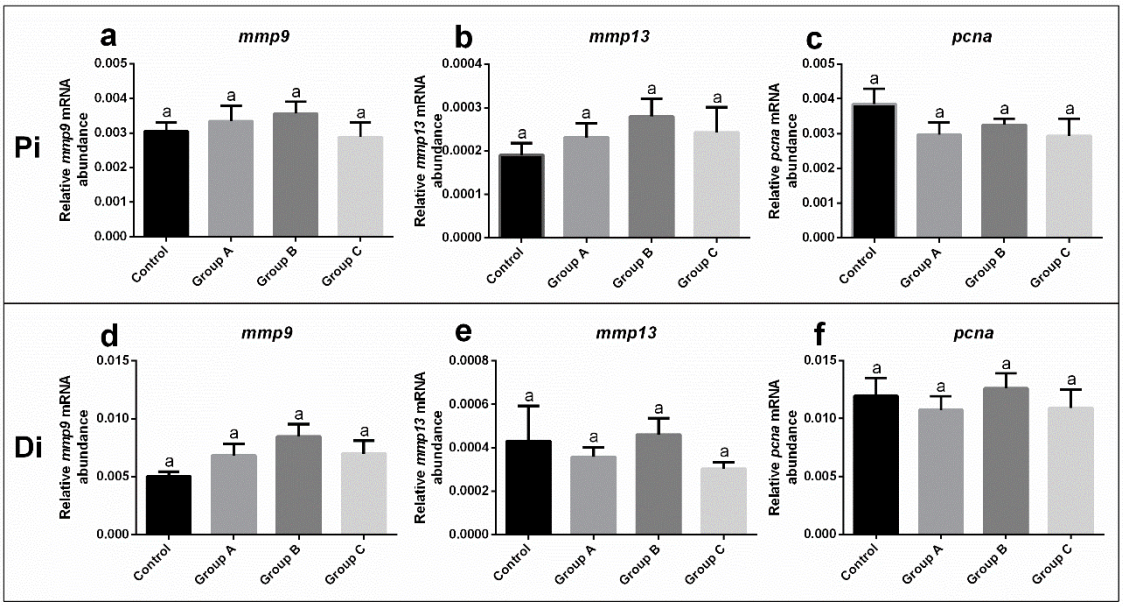

Fig. 5. Relative mRNA abundance of genes involved in tissue recovering (*mmp9*, *mmp13* and *pcna*) analysed in *O. latipes* proximal (Pi) and distal intestine (Di), fed a commercial diet (Control), a challenge diet (Group A) and challenge diets including 5% of *S. latissima* (Group B) and 5% of *P. palmata* (Group C), sampled at the end of the experiment (25 days). Values are presented as mean ± SEM (n = 10). No significant differences were observed among experimental groups ($p > .05$), as indicated by the letter above each column.

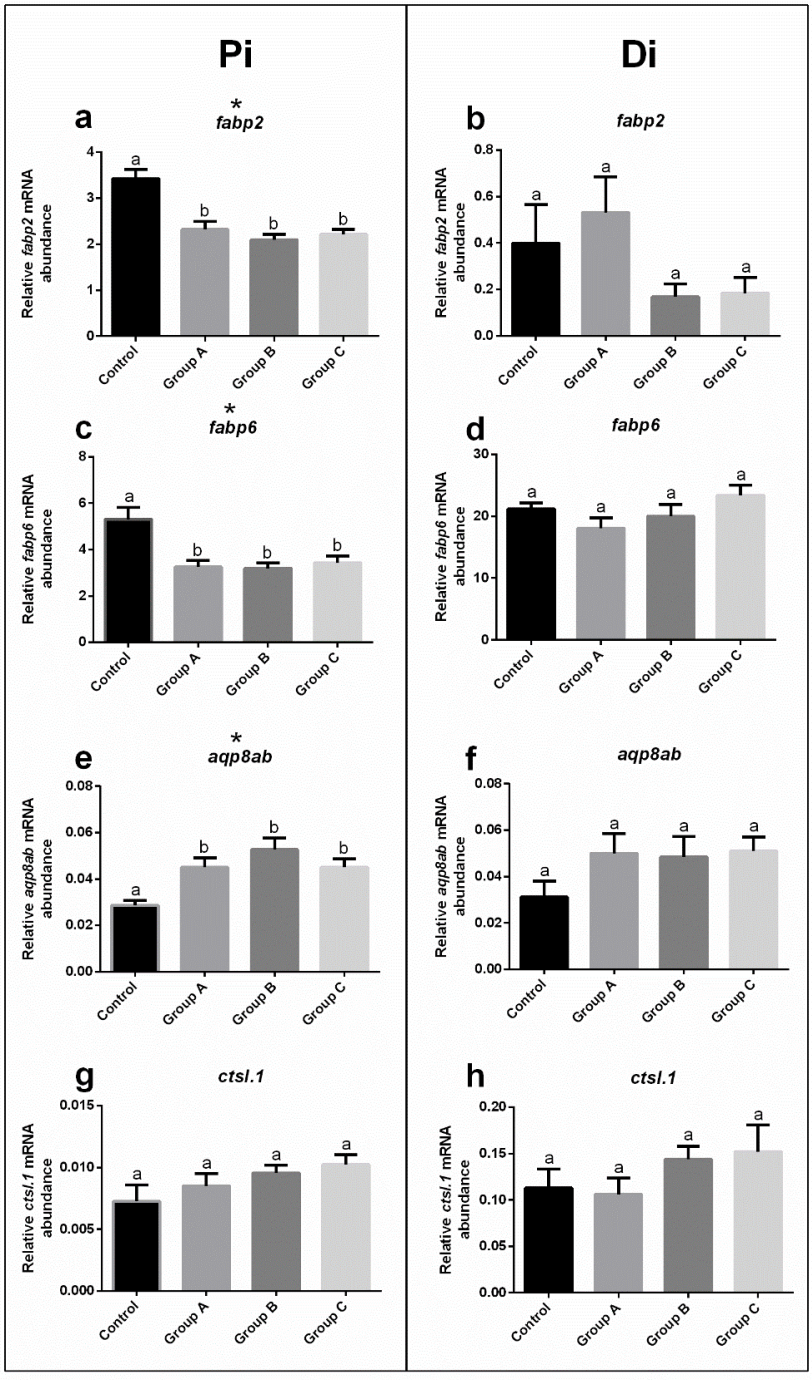

Fig. 6. Relative mRNA abundance of genes involved in normal gut function typically affected in SBM-induced enteritis (*fabp2*, *fabp6*, *aqp8ab* and *ctsl.1*) analysed in *O. latipes* proximal (Pi) and distal intestine (Di), fed a commercial diet (Control), a challenge diet (Group A) and challenge diets including 5% of *S. latissima* (Group B) and 5% of *P. palmata* (Group C), sampled at the end of the experiment (25 days). Values are presented as mean ± SEM (n = 10). Different letters above each column indicate statistical differences among experimental groups ($p < 0.05$).

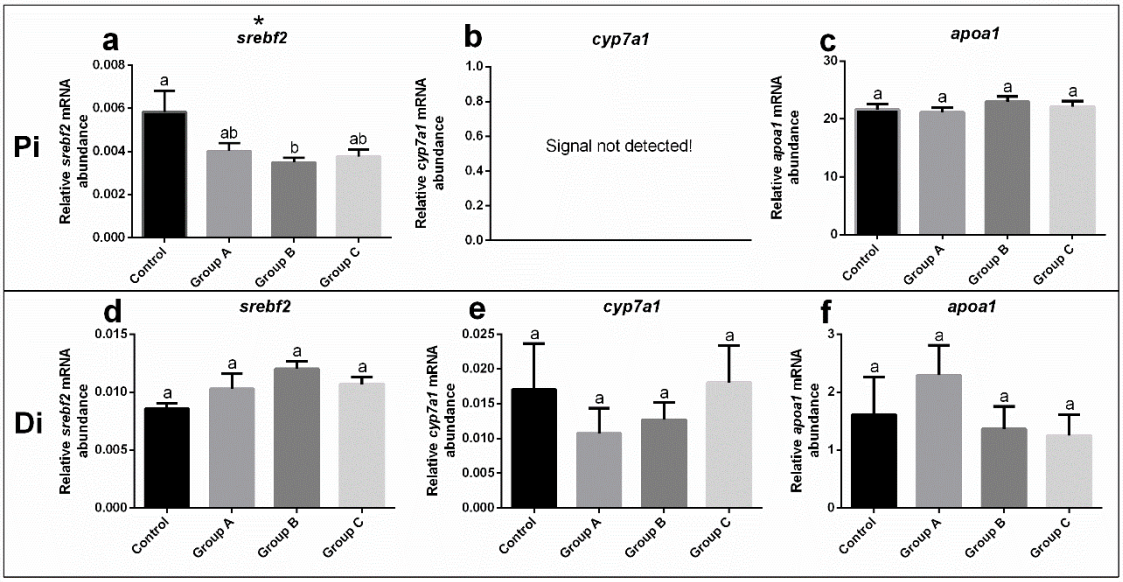

Fig. 7. Relative mRNA abundance of genes involved sterol metabolism (*srbf2*, *cyp7a1* and *apoa1*) analysed in *O. latipes* proximal (Pi) and distal intestine (Di), fed a commercial diet (Control), a challenge diet (Group A) and challenge diets including 5% of *S. latissima* (Group B) and 5% of *P. palmata* (Group C), sampled at the end of the experiment (25 days). Values are presented as mean ± SEM (n = 10). Different letters above each column indicate statistical differences among experimental groups ($p < 0.05$).

Discussion

Intestinal inflammation due to the use of alternative feed ingredients to meet the growing demand of aquaculture fish is a major challenge in aquafeeds (Krogdahl and Bakke 2015). The aim of the present study was to explore medaka as a model for feed-induced intestinal inflammation and assessing the possible functional effects of two macroalgae as feed additives. To that aim, diet formulations in the present study included high levels of soybean meal (35%), purified soy saponins (0.5%) and cholesterol (3%) to induce an intestinal inflammatory response, as previously observed in several other fish species (Chen et al. 2011; Hedrera et al. 2013; Krogdahl et al. 2015; Gu et al. 2016; Coronado et al. 2019). Gut health was assessed through gut histology, stress and immune response as a response to inflammation, as well as evaluation of genes involved in cholesterol metabolism.

In the present study, after 25 days of dietary treatment, a significantly lower fish wet weight was observed in fish fed the challenge diets (Group A, B and C) compared to the Control diet. Not surprisingly, observed, although not quantified, differences in feed intake were also evident. Different nutritional quality of the commercial feed (not provided) in contrast to the formulated feeds may explain differences in feed intake and fish weights. The lower fish weight may also be due to possible negative effect of the challenge diets, as similar responses have been reported previously in fish challenged with plant-based feeds (Bonaldo et al. 2011; Yu et al. 2019). However, overall gut histology and gene expression results did not show clear negative effects. To the author's knowledge, this is the first study assessing possible intestinal inflammation triggered by feed intake in the whole medaka genus and comparisons are therefore difficult to make.

In other fish species, that include Atlantic salmon, rainbow trout, turbot, Japanese flounder, common carp and zebrafish (Krogdahl et al. 2003; Urán et al. 2008; Yamamoto et al. 2008; Chen et al. 2011; Gu et al. 2016; Coronado et al. 2019), SBM has been observed to cause an inflammatory response in the distal intestine. This pathology is characterized by a shortening of the mucosal folds, widening of the lamina propria and subepithelial mucosa and a strong infiltration of various inflammatory cells, along with physiological and molecular alterations of cholesterol and bile salt absorption and metabolism (Chen et al. 2011; Krogdahl et al. 2015; Gu et al. 2016; Coronado et al. 2019).

In the present study, however, none of the above conditions were detected by histological analysis, which revealed no alterations in the Pi or Di mucosa in terms of inflammatory influx, morphology and integrity of intestinal folds after 25 days of dietary treatment. Furthermore, these results were supported by gene expression analysis of immune genes including pro-inflammatory and anti-inflammatory markers as well as by stress response and tissue recovering markers, which did not show any significant alteration among experimental groups. These results were also partially supported by the analysis of genes involved in normal gut function.

Thus, our results may indicate that the medaka showed tolerance to the SBM (35%) and pure saponin (0.5%) levels tested in the present study, as recently reported for juvenile turbot (Gu et al. 2016) and zebrafish larvae (Hedrera et al. 2013; Fuentes-Appelgren et al. 2014; Coronado et al. 2019), where high SBM inclusion levels are needed to induce intestinal inflammation. In Atlantic salmon, inclusion levels as low as 10% SBM in the diet during 60 experimental days elicited detrimental inflammatory effects at the intestinal level (Krogdahl et al. 2003; Urán et al. 2009). In juvenile turbot, another carnivorous fish, a 22% SBM inclusion in the diet did not affect the digestibility and intestinal histology after 77 experimental days (Bonaldo et al. 2011), while higher inclusion levels (26–54%) induced enteritis in the distal intestine comparable to that found in other carnivorous fish species such as salmon (Gu et al. 2016) . Similarly, in zebrafish larvae a 30% SBM dietary inclusion did not affect granulocyte infiltration and immune response gene expression (Fuentes-Appelgren et al. 2014), whereas higher inclusion levels (50%) induced a clear inflammatory response after only 2 days (Hedrera et al. 2013; Fuentes-Appelgren et al. 2014; Coronado et al. 2019).

The key anti-nutrient responsible for feed-induced enteritis in fish was recently confirmed to be saponins (Krogdahl et al. 2015). Saponins occur naturally in legumes and other seed crops such as soy, pea, sunflower, and lupin (Rajauria 2015). Their effects have been tested in several fish species and divergent effects have been reported in response to the dietary inclusion levels, as well as fish species. At low levels, saponins have been shown to positively modulate the immune system in Japanese flounder and juvenile turbot (Chen et al. 2011; Yu et al. 2019). However, at higher levels, sing of enteritis commonly appeared in several fish species. These levels ranged from 2% to 4% in salmon (Krogdahl et al. 2015), 3.3% in zebrafish larvae (Hedrera et al. 2013; Fuentes-Appelgren et al. 2014; Coronado et al. 2019), 3.2% to 6.4% in Japanese flounder (Chen et al. 2011) and 2.5 to

15% in turbot (Gu et al. 2018). In the present study, 0.5% purified saponins were added in the diet and estimated saponin levels ranged probably around 2.25%, considering that the 35% SBM included in the diet was equivalent to an estimated 1.75% saponins (Krogdahl et al. 2015). Therefore, it is possible that the saponin dietary inclusion level tested (around 2.25%) in the present study was not enough to stimulate an inflammatory response in O. latipes.

As regards the analysed genes involved in normal gut function, significant differences in gene expression among the different diet groups were detected only in Pi samples. Signs of SBM-induced enteritis in fish have generally been observed in the Di. Nevertheless, results did not show a clear intestinal damage. As regards *fabp2* and *fabp6*, markers involved in lipid uptake and transport, indicative of intestinal barrier injury (fabp2 and fabp6) (Krogdahl et al. 2015; Aghaallaei et al. 2016), higher expression levels in the challenge groups (Group A, B and C) with respect to Control were observed, indicating injury of enterocytes and impaired lipid transport in fish fed feeds containing SBM and saponins. This kind of damage is usually accompanied by altered permeability which may lead to impaired digestive functions (Madsen et al. 2014; Krogdahl et al. 2015; Hu et al. 2016). However, in the present study, the *aqp8ab* gene expression was highly expressed in the in the challenge groups with respect to Control, suggesting a general welfare as regards intestinal water transport.

Another effect associated with SBM and saponin intake, in A. salmon, involves alterations of cholesterol and bile salt absorption and metabolism (Krogdahl et al. 2010). As regards SBM, soy saponins are implicated in the formation of non-absorbable complexes due to their amphiphilic property which have the ability to bind and form complexes with cholesterol (Glauert et al. 1962; Malinow et al. 1977). This effect has the ability to increase the excretion of cholesterol, which in turn activates the molecular mechanisms for cholesterol biosynthesis, sterol efflux by enterocytes, and suppression of sterol uptake from the intestine (Kortner et al. 2013, 2014). The net result translates in increased export and reduced import of cholesterol. A key marker of cholesterol biosynthesis and transport is the sterol regulatory element binding transcription factor 2 (*srebf2*) (Mullen et al. 2004). In the present study, challenged fish (Group A, B and C) showed a lower level in *srebf2* gene expression compared to the Control fish. This may be explained by the fact that these diets also included a high dietary cholesterol content (3%) which could counteract the increasing cholesterol biosynthesis effect of SBM. As

regards *cyp7a1*, a rate-limiting enzyme in primary bile acid synthesis by the liver, relevant in cholesterol catabolism (Howarth et al. 2010), no detectable gene expression was detected in Pi samples and weak signals in Di samples. *Apoa1* is a gene that codes for the main protein of high-density lipoproteins, which has been implicated in the reverse transport of excessive cholesterol from peripheral tissues to the liver via excretion of bile acids. No significant differences among experimental groups were observed for this gene. In sum, no clear effects on sterol metabolism were observed due to SBM and saponin intake, but there were indications of a limited regulation of cholesterol synthesis as a response to the dietary cholesterol supplemented.

Seaweeds represent an interesting protein source (Rajauria 2015) and they possess a number of metabolites which confer them functional properties such as antibacterial, antioxidant, anti-inflammatory and antiviral properties. A number of studies have demonstrated immunomodulatory effects at low seaweed dietary inclusion, 5% or lower (Sotoudeh and Jafari 2017; Shi et al. 2019; Wang et al. 2019). In the present study, a possible protective effect of seaweeds on intestinal immune response was assessed. However, none of the analysed markers showed significant differences linked to the seaweed administration, except for a significant reduction of *srebf2* gene expression in the fish fed diets including *S. latissima* (Group B) with respect only to Control. While reductions in *srebf2* gene expression may be indicative of reduced cholesterol biosynthesis induced by *S. latissima*, a possible nutritional effect of the commercial feed (Control) is not ruled out. Thus, even if the challenge diets did not induce any clear inflammatory reaction, the present study could suggest no immunomodulatory effects associated to *S. latissima* and *P. palmanta* intake. However, more studies are needed to discard possible immunomodulatory effects in *O. latipes*.

In the present study, the distinction into proximal and distal intestine in medaka was not obvious and limited information on the medaka gut morphology was available. In other fish species, such as salmon and zebrafish, this distinction has been characterized based on gut morphology (Wallace et al. 2005; Løka et al. 2013). In medaka, the gut, which is thin and fragile compared to salmon and zebrafish, showed a high length variability and a high degree of looping (Iwamatsu 2012; Aghaallaei et al. 2016). These characteristics made it difficult to distinguish the Pi from the Di. Therefore, the present study followed the gut subdivision proposed by Aghaallaei et al. (2016), which divided the medaka gut into six equal parts and found morphological and transcriptomic resemblance to the small

and large intestine of mammals associated to the first four and the last fifth and sixth sections, respectively. The present study supported the described subdivision, with a gene expression that correlated with specific markers for the small and large intestine. In particular, apoa1 and fabp2 showed a higher magnitude in the gene expression of Pi samples, while fabp6 and ctsl.1 showed a higher magnitude in the gene expression of Di samples, as it would be expected from small and large intestine.

In conclusion, *O. latipes* seemed to be tolerant to the SBM and saponin levels tested in the present study. Histological evaluations did not show any pathological signs in the proximal or distal intestinal mucosa after 25 days of dietary treatment. Gene expression analysis did not show significant variations in genes involved in stress and immune response, tissue recovering, as those known in SBM-induced enteritis in Atlantic salmon. Genes involved in normal gut function and sterol metabolism showed significant variations due to the challenge diets but nevertheless they did not indicate a clear inflammatory response. Seaweed supplementation did not induce any effect on fish growth and gut health parameters. This was the first feeding study assessing possible intestinal inflammation triggered by feed intake in the medaka genus. Limitations of the present study included a limited evaluation of markers and it is possible that markers that are more sensible could detect signs of enteritis. Further studies should test higher SBM and saponin inclusion levels and apply techniques that are more sensitive by using other approaches such as proteomics analysis.

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General conclusions

A major goal of the aquaculture research is to replace fish meal and fish oil for aquafeeds with more sustainable cost-effective, and environmental friendly ingredients ensuring fish health and welfare standards. Due to minimal environmental impact, compared with most conventional feed commodities, insects and seaweeds deserve a growing attention as candidate ingredients for aquafeeds. Early research on insect meal and seaweed as feed additives is promising but in its infancy. Presently, it is largely unknown the effects of these unconventional feed ingredients on the fish biological responses.

In that regard, the present thesis evaluated the effects of insect based diets and seaweeds on key fish biological responses, improving the understanding of the mechanisms involved in fish nutrition and growth. A comprehensive knowledge on the effects of these innovative feed ingredients on the fish responses at the histological, biochemical, physiological and molecular level was achieved through a multidisciplinary approach. Taking advantage of powerful fish models, that included zebrafish as a freshwater fish, clownfish as a marine one, and medaka as brackish species, information obtained in the present thesis may be used to generalize how several biological processes occur in related organisms and be applied to finfish production.

The first chapter opened the possibility for the use of a more sustainable ingredient that consisted of a 100% insect diet (*H. illucens*), in the larval rearing of zebrafish. Results were promising and demonstrated that the new diet promoted good fish performances with no adverse effects on fish health. In addition, results also highlighted the importance of the insect rearing substrate in modulating the insect nutritional value. Further studies should be considered applying lower insect meal inclusions because of their possible role as bioactive molecules and immunomodulators.

The second chapter followed-up previous observations and therefore it tested, for the first time, partial dietary inclusion of *H. illucens* meal in zebrafish larval diets. Results supported previous observations, demonstrating good fish performances. However, a 50% fish meal replacement by *H. illucens* affected both lipid composition and accumulation of the larvae. Further studies (including a long-term exposure) are needed to better understand the physiological responses of fish, including very low *H. illucens*-meal substitutions, which may play an immunomodulatory role as well as possible

modifications of the growing substrate to evaluate induced variations in insect lipid profile.

The third chapter tested partial dietary inclusions of *H. illucens* meal in the marine fish *A. ocellaris*. This was the first study on a marine ornamental fish species. Unlike the previous observations in chapter one and two, clownfish fatty acid was strongly affected by the novel ingredient at both biochemical and spectroscopic level. Accordingly, important alterations in the topographical distribution of lipids, fatty acids, proteins and glycogen in the liver were detected by Fourier Transform Infrared Microspectroscopy. Surprisingly, the fish performance was not affected. Thus, this study opened the possibility of using insect based diets in the juvenile culture of marine ornamental fish. However, more studies are needed to better understand the physiological responses of clownfish fish over a longer period of time to evaluate possible effects on the different stages of their life cycle, especially on the reproductive stage.

Finally, the fourth chapter evaluated, for the first time in the whole *Medaka* genus, the possible use of medaka (*Oryzias latipes)* as a model for food-induced intestinal inflammation, and the possible functional effect of seaweeds as feed additives. Results revealed tolerance of *O. latipes* towards soybean meal and saponins, ingredients known to induce intestinal inflammation in aquaculture species such as Atlantic salmon. Seaweed supplementation did not induce any effect on fish growth and gut health parameters but further studies with more sensitives techniques are encouraged to reveal possible subtle effects. Further studies should test higher soybean meal and saponin inclusion levels in medaka diets.

In summary, the present PhD project provided insights about the physiological and nutritional mechanisms involved in the larval/juvenile phase of fish fed insect meal and feed additives, that were completely unknow before the completion of this thesis. However, information about the effects of insect-based diets over the whole life cycle of fish is presently missing and more studies are therefore needed. This work highlighted the important role that insects may play in the circularity of natural resources, that, if applied to the aquaculture sector, has the potential to improve the economic benefits while encouraging sustainability and competitiveness in the long term.

Tangible outcomes derived from the present thesis. The first 3 chapters have already been published in high impact international peer-reviewed journals. The already 12 scientific

citations of the first chapter by peer-reviewed papers by September 2019, 7 citations of the second chapter and 7 of the third chapter highlight the relevance of the present thesis (google scholar). This work has been disseminated in national and international congresses and impacted not only the scientific community, but also the general public through expos, conferences and seminars, demanding collective awareness on sustainability and the impact of current aquaculture practices.

Appendix

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Rearing Zebrafish on Black Soldier Fly (*Hermetia illucens*): Biometric, Histological, Spectroscopic, Biochemical, and Molecular Implications

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Abstract

A desirable goal of the aquaculture sector is to replace most of fish meal and fish oil with more sustainable, cost-effective, and environmental friendly ingredients ensuring fish health and welfare standards. Due to minimal environmental impact, compared with most conventional feed commodities, insects deserve a growing attention as candidate ingredients for aquafeeds. The present study investigated, for the first time, the possible application of a 100% insect diet in zebrafish larval rearing. Through a multidisciplinary approach, the major biological responses of fish to the new diets were assessed. Results of biometry, fatty acid composition, expression of genes involved in fish growth, stress response, lipid metabolism, chitinolytic activity, gut inflammation, and liver macromolecular composition suggested a possible application of insect larvae for zebrafish larval rearing. However, further studies are necessary to better understand the use of this insect species in the rearing of fish.

Keywords: black soldier fly, gene expression, fatty acid profile, insect diet, aquaculture

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Original Article

Partial Dietary Inclusion of *Hermetia illucens* (Black Soldier Fly) Full-Fat Prepupae in Zebrafish Feed: Biometric, Histological, Biochemical, and Molecular Implications

Matteo Zarantoniello,[1] Leonardo Bruni,[2] Basilio Randazzo,[1] Arturo Vargas,[1] Giorgia Gioacchini,[1] Cristina Truzzi,[1] Anna Annibaldi,[1] Paola Riolo,[3] Giuliana Parisi,[2] Gloriana Cardinaletti,[4] Francesca Tulli,[4] and Ike Olivotto[1]

Abstract

Due to minimal environmental impact, compared to most conventional feed commodities, insects deserve a growing attention as candidate ingredients for aquafeeds. This study tested, for the first time during zebrafish larval rearing, the effects of an increasing replacement (0%–25%–50%) of fish meal by black soldier fly (BSF) full-fat prepupae meal. All diets were formulated to be isonitrogenous and isolipidic. A multidisciplinary approach, including biometrics, histology, gas chromatography–mass spectrometry, and molecular analyses, was applied to better understand the biological responses of larval zebrafish to the different partial inclusions of BSF in the feed. Generally, results are promising, but a 50% of BSF meal inclusion in the diet affected both lipid composition and accumulation in the larvae.

Keywords: black soldier fly, gene expression, fatty acid profile, insect diet, aquaculture

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Insect meal based diets for clownfish: Biometric, histological, spectroscopic, biochemical and molecular implications

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ARTICLE INFO

Keywords:
Black soldier fly
Gene expression
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ABSTRACT

Basic nutrition for tropical fish is undeniably lacking, mainly due to the concentration of research efforts on and funding for food fish species. However, as the marine ornamental aquaculture industry continues to grow and expand, it is now essential to direct nutritional research to mitigate the current lack of information related to feeds specifically formulated for ornamental fish. Due to a minimal environmental impact, compared to most conventional feed commodities, insects deserve a growing attention as candidate ingredient for aquafeeds and *Hermetia illucens*, the black soldier fly, is presently one of the most promising insect species. The aim of the present study was to formulate new insect based diets and to evaluate, through a multidisciplinary approach including morphological, biochemical, spectroscopic and molecular analysis, the major biological responses of *Amphiprion ocellaris* juveniles fed on these diets over a 106 days experimental period. Biometry did not reveal differences in survival and growth; fish fatty acid composition was influenced by the diets. Real-Time PCR analyses on genes involved in fish growth (*igf1*, *igf2* and *mstn*), lipid metabolism (*ppara* and *pparβ*) and stress response (*gr* and *hsp70*), did not show significant differences among groups. Histology showed a normal intestinal mucosa and liver parenchyma in all experimental groups. Lower amounts of lipids and proteins, together with a higher amount of glycogen, were detected by FTIR in the liver of clownfish fed on diets including higher insect meal amounts.

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SCIENTIFIC REPORTS

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

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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 months 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-months feeding on Black Soldier Fly based diets did not show major negative effects on zebrafish. Gut 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.

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The influence of diet on the early development of two seahorse species (*H. guttulatus* and *H. reidi*): Traditional and innovative approaches

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ARTICLE INFO

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Seahorse
Live prey
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ABSTRACT

Larval nutrition plays a key role in the development of a sustainable aquaculture where fish development, health and wellness are of prime importance. For some species, satisfactory growth and survival rates are met providing exclusively enriched rotifers and *Artemia*; however, feeding on copepods during the larval period has been shown to improve growth in both larval and juvenile fish, including seahorses. For the first time, the effects of different diets (*Artemia* and copepods) on the early development of juvenile seahorses (*H. guttulatus* and *H. reidi*) development were analysed by combining biometry, traditional histology and FPA-FTIR Imaging spectroscopy. Survival and growth and biochemical composition on the liver in seahorse were significantly affected by the type of diet offered. The results achieved were related to differences in the digestion of the two types of live preys, mainly dependent on their biochemical composition and permeability of the exoskeleton.

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ORIGINAL ARTICLE

WILEY Aquaculture Research

Kluyveromyces fragilis RNA extract supplementation promotes growth, modulates stress and inflammatory response in zebrafish

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Abstract

Due to the undesirable consequences associated with antibiotics use, researchers and food producers have studied alternative feeding, for the control of fish diseases and animal welfare improvement. However, the beneficial properties of RNA yeasts extract in aquaculture have been barely considered. The present study investigated the beneficial properties of RNA yeast extract from *Kluyveromyces fragilis* on survival rate, weight and length as well as on molecular pathways involved in growth, immuno-system and oxidative stress using zebrafish (*Danio rerio*) as an experimental model. The yeast extract has been administrated to zebrafish at three different concentrations (60–180–300 ppm) via zooplankton (*Artemia salina*) for 21 days. Results highlighted yeast extract RNA capability to enhance growth and to improve larvae survival rate in a dose-dependent manner. In fact, gene expression data showed the ability of the RNA yeast extract to up-regulate genes involved in growth and to restore stress-related condition due to the early larval development coupled with the RNA yeast extract administration. In addition, gene expression showed that RNA yeast extract acts as inflammatory-reducer, but did not enhance the immune response. Histological analysis showed that all three treated groups displayed a thicker gut epithelium, higher intestinal crypts coupled with higher enterocytes lengths compared to control group. In conclusion, these findings provide a large gene network through which yeast RNA extract acts and, by inducing transcriptional changes, modulate the physiological control of growth and inflammatory response coupled with the increase in length and dry weight, together with a higher survival ratio and an intestinal architecture amelioration.

KEYWORDS
growth, immune system, yeast RNA extract, zebrafish

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Feeding the lined seahorse *Hippocampus erectus* with frozen amphipods

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Ingestion rates

ABSTRACT

Amphipods are a natural and alternative feed in aquaculture but their potential use is limited by lack of knowledge of feeding regimes. The present study provides a feeding regime of frozen amphipods (*Elasmopus pectenicrus*) for the lined seahorse *Hippocampus erectus*, the second most traded species worldwide. Ingestion rates of amphipods were measured to establish the optimal amphipod size, duration of feeding and feed ration. Use of large amphipods (8.7 ± 1.1 mm total length) increased the total ingested biomass in fish of 60–110 mm standard length, compared to medium and small amphipods, in accordance with previously suggested relative sizes. The highest feeding activity occurred during the first 12 min. Fish ingested 4.5 ± 2.3% their wet weight, which is similar to the feed ration previously proposed for other seahorse species. The present study supports the potential of amphipods as a feed in aquaculture and will aid their use in culture protocols.