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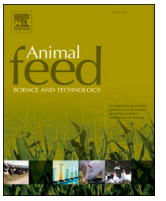

Insect-based aquafeeds modulate the fatty acid profile of zebrafish: A comparison on the different life stages

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ABSTRACT

Insects are considered as an alternative and sustainable ingredient for feed production. In this study, *Hermetia illucens* (Hi) prepupae (fed on coffee roasted by-product added with 10 % *Schyzochytrium* sp., a marine protist rich in polyunsaturated fatty acids) was processed into meal and included at graded levels in five experimental diets to replace dietary fish meal (0, 25, 50, 75, 100 %) offered to zebrafish (*Danio rerio*) as experimental model. The fatty acids (FAs) profile of fish larvae (20 days), juveniles (2 months, deprived of the viscera) and adults (6 months, male and female, deprived of the viscera) specimens was investigated to evaluate the impact of dietary FM replacement with full-fat Hi prepupae meal. For the first time, the quantification of FAs in *Danio rerio*, performed by gas chromatography-mass spectrometry, was computed in absolute terms (mg 100 g^{-1} dw), identifying the real variation in the content of any single FA. In fish, quantified total FAs in larvae, juvenile, adult male and adult female were 134 ± 2, 235 ± 8, 266 ± 3 and 266 ± 8 mg g^{-1} dw, respectively. With respect to zebrafish fed the control diet, specimens fed diet with increasing level of Hi prepupae meal showed significantly higher content of saturated and omega6 FAs, and significantly lower content of poly-unsaturated and omega3 FAs, reflecting partially the FAs composition of the administered diets. At the same time, the docosahexaenoic/eicosapentaenoic acid ratio increased significantly. Moreover, adult female showed a higher content of PUFA with respect to adult male, which could be justified by the needs of physiological reproductive processes. The principal component analysis demonstrated that the FAs composition in *Danio rerio* depends on both the life stage and the diet.

1. Introduction

Aquaculture is the fastest growing food production sector in the world (Naylor et al., 2021), and it is estimated that about 62 % of the sea food will come from aquaculture in 2030 (FAO, 2022). Aquaculture sector has two important potentials: firstly, to respond promptly to the increased demand for animal proteins by the growing world population and, in addition, to alleviate the pressures on

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wild stocks, which are now severely reduced (FAO, 2022). A further advantage of aquaculture is related to the possibility to modulate the nutritional quality of farmed fish respect to the wild counterpart (Cahu et al., 2004). Aquaculture, however, raises concern about the impact on global food resilience as it competes for crop and marine resources with livestock, the energy industry and even direct human consumption (Gasco et al., 2018; Eroldoğan et al., 2022). From these considerations it is clear how the aquaculture industry must focus attention on the identification of new and more sustainable aquafeed ingredients respect to FM and FO by maintaining the fish quality traits (Thilsted et al., 2016).

Among the various alternatives proposed, the possibility of using insects as sustainable sources of protein for aquaculture has gained increasing attention, thanks to their reduced environmental footprint (Craig Sheppard et al., 1994; Randazzo et al., 2021b; Zarantoniello et al., 2022). In fact, insects have limited emissions of greenhouse gases, a high feed-conversion efficiency, limited water and space needs (Oonincx et al., 2010; van Huis and Oonincx, 2017; Akhtar and Isman, 2018). Moreover, most insects can grow on organic substrates deriving from by-products of the agro-food industry, which could reduce the problems associated with the disposal of waste products from production, with a view to developing an eco-sustainable circular economy (Henry et al., 2015; Gasco et al., 2016; Barragan-Fonseca et al., 2017; Vargas-Abúndez et al., 2019; Harsányi et al., 2020; Wang et al., 2020). However, insects are characterized by a lipid profile not suitable for the nutritional needs of fish (and those marines in particular), as rich in saturated fatty acids (SFA) and lacking in polyunsaturated fatty acids (PUFA) (St-Hilaire et al., 2007; Ushakova et al., 2016; Barragan-Fonseca et al., 2017; Zarantoniello et al., 2018). PUFA deficiencies in fish are associated with a number of negative effects, such as reduced growth, hepatic steatosis and myocarditis, increased mortality, incorrect pigmentation, disruption of branchial epithelia, reduced fertility and fertilization rate (Randazzo et al., 2020; Tocher, 2010; Chemello et al., 2022). PUFA also have beneficial effects on human health, such as prevention and treatment of neurodegenerative diseases such as Alzheimer's and Parkinson's, cardiovascular diseases, but also diabetes, cancer, depression, mental illness (Sidhu, 2003; Simopoulos, 2008; Benjamin et al., 2017; Zárate et al., 2017; Shahidi and Ambigaipalan, 2018). Therefore, it is important to take foods rich in PUFA, and among the foods richer in these FAs, there is fish.

In 2015, the European Food Safety Authority (EFSA) has drawn up a list of insect species potentially usable in feed and food production in the EU, including *Hermetia illucens* (Hi, Diptera, Stratiomydae) (EFSA, 2015). The breeding of Hi has been proposed as an efficient way to convert organic waste into biomass with a high nutritional value for various purposes, including feed (Nguyen et al., 2015). Hi, like all insects, is poor in PUFA (St-Hilaire et al., 2007; Ushakova et al., 2016; Barragan-Fonseca et al., 2017), but its lipid profile can be modulated by the growing substrate. Several substrates enriched with PUFA have been tested to obtain pre-pupae Hi enriched with unsaturated FAs (Tomberlin and Sheppard, 2002; Nguyen et al., 2013; Barroso et al., 2014). In the paper of (Truzzi et al., 2020a), the addition of 10 % (wet weight) of the marine protist *Schizochytrium* sp. to the larval growth substrate (coffee siverskin, a coffee roasting by-product) of Hi has been found to be a good method to obtain PUFA-enriched Hi prepupae. In turn, the Hi prepupae thus obtained have been studied as a substitute for FM and FO in diets for the breeding of zebrafish (*Danio rerio*) (Zarantoniello et al., 2020a, 2020b, 2021). Both Hi prepupae and Hi-based diet did not present a chemical risk for the health of zebrafish specimens (Truzzi et al., 2020b, 2022).

Zebrafish is an ideal organism to better understand fish physiological responses to new ingredients because of its short life cycle, high reproductive rate, well-defined developmental processes, and abundant information on its genomic features, like that of vertebrates, including humans (Grunwald and Eisen, 2002; Dahm and Geisler, 2006; Egan et al., 2009; Harris et al., 2014; Hoo et al., 2016). In addition, zebrafish has been recently recognised as a useful model organism for fish nutrition studies to be applied in the aquaculture sector (Piferrer and Ribas, 2020).

In general, studies about lipid profile in *Danio rerio*, or in fish in general, measure FAs content as percentage of total FAs. In such measurement, the variation of each individual FA depends on the percentage modification of other FAs, but it does not identify their absolute variation, which would be more useful to better clarify the influence of external factors on lipid profile variations in fish. No study so far has quantified FAs in the muscle of *Danio rerio* in terms of mass unit per tissue weight.

The aim of this research is to study the absolute content of FAs in the experimental model *Danio rerio* during its whole life cycle, in relation to the FAs composition of the feed and to the life stage of fish (larvae, juvenile, adult male and female stage) This work is part of the research project NUTRIFISH, to study the possibility of including Hi, grown on substrates suitably enriched with PUFA, in feed intended for aquaculture.

2. Materials and methods

2.1. Ethics

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

2.2. Insects rearing

The *Hermetia illucens* (Hi) feeding substrate was prepared using a coffee industry by-product (coffee silverskin; moisture 44 %) as the main component according to Zarantoniello et al. (2020a) and summarized as follows. Firstly, coffee silverskin (Saccaria Caffe` S.R. L., Marina di Montemarciano, AN, Italy) was grounded to 2 ± 0.4 mm particle size and then 10 % (w/w) of *Schizochytrium* sp. (provided freeze-dried by AlghItaly Società Agricola S.R.L., Sommacampagna, VR, Italy) was added. A final moisture of ~70 % was reached by adding distilled water to the feeding substrate. Six-day-old Hi larvae (provided by Smart Bugs s.s. Ponzano Veneto, TV, Italy) were individually counted and divided into groups of 640 specimens per replicate (n = 65 for a total of 41'600 Hi larvae) and

maintained following the rearing conditions reported by Zarantoniello et al. (2020a). The feeding rate per larva was calculated at 100 mg/day according to Diener et al. (2009) and maintained constant during the larval growth by adding new feeding substrate once a week (448 g for each box) after the removal of the old rearing substrate. Once the prepupal stage, identified by the change in the tegument colour from white to black (Milanović et al., 2021), was reached, insects were collected, washed, dried and stored at − 80 °C.

2.3. Fish diet production

The ground freeze-dried full-fat Hi prepupae were prepared using a Retsch Centrifugal Grinding Mill ZM 1000 (Retsch GmbH, Haan, Germany) as previously described in Zarantoniello et al. (2021). A control diet (Hi0) containing fishmeal (FM), a vegetable protein mixture (wheat gluten and pea protein concentrate) and fish oil (FO) as major ingredients, was prepared according to a commercially available standard diet for zebrafish (Zebrafeed, Sparos ltd, Olhão, Portugal). Four experimental Hi-based diets were prepared by including graded levels of PUFA-enriched full-fat Hi prepupae meal to replace FM on weight-to-weight basis with the Hi0 formulation (approximatively 25 %, 50 %, 75 % and 100 % of FM substitution, named Hi25, Hi50, Hi75, and Hi100, respectively). The ingredients and the proximate composition of the experimental diets are shown in Table 1. The experimental diets were formulated to be grossly isonitrogenous (N x 6.25: 50.9 ± 0.5 %) and isolipidic (ether extract: 13.3 ± 0.6 %) as previously described in Zarantoniello et al. (2021).

2.4. Biometry

For growth measurements, fish were randomly collected from the different tank at hatching (3dpf), at 20, 60, and 180 dpf, for larvae, juvenile and adult specimens, respectively. Larvae were measured and weighed as a pool of five *per* tank (Zarantoniello er at, 2020a), whereas juvenile and specimens were individually considered (Zarantoniello et al., 2020b; Chemello et al., 2022). The standard length was determined by a sliding calliper (precision, 0.1 mm), and the weight was measured by an analytical balance (precision 0.1 mg, OHAUS Explorer, OHAUS Europe GmbH, Greifensee, Switzerland). Specific growth rate was calculated for larvae and juvenile as follows: SGR % = [(lnWf – lnWi)/t) × 100, where Wf is the final wet weight, Wi, the initial wet weight, and t, the number of days (Zarantoniello er at, 2020a).

2.5. Experimental design

Zebrafish larvae were initially reared in fifteen 20 L tanks to set up the five experimental dietary treatments (3 tanks per experimental groups with 500 larvae per tank) (Zarantoniello et al., 2020a). Details on zebrafish rearing are reported in Supplementary Material file. Starting from 5 dpf to 6 months, zebrafish were fed on the experimental diets as follows: Hi0 group: fish fed diet containing 0 % of full-fat Hi prepupae meal; Hi25, Hi50, Hi75 and Hi100 groups: fish fed diets including 25 %, 50 %, 75 % and 100 % of full-fat Hi prepupae meal respect to FM respectively. Feed particle sizes were < 100 μm from 5 to 15 dpf, 101–200 μm from 16 to 30 dpf and 201–400 μm from 31 to 60 dpf and 401–600 μm from 61 until the end of the experiment. Zebrafish were fed the experimental diets (2 % body weight, BW) twice a day, and all the feed was consumed by the fish. In addition, from 5 to 10 dpf, all groups were fed (one

Table 1
Ingredients (g Kg^{-1}) and proximate composition (g 100 g^{-1}) of the experimental diets.

	Hi0	Hi25	Hi50	Hi75	Hi100
Ingredients (g kg^{-1})					
Fish meal[1]	470	400	250	110	-
Vegetable protein mix[2]	220	230	298	385	440
BSF prepupae meal	-	115	235	350	460
Wheat flour[3]	198	172	120	110	72
Fish oil	80	51	25	10	-
Soy lecithin	8	8	8	11	4
Mineral and Vitamin supplements[$]	14	14	14	14	14
Binder	10	10	10	10	10
Proximate composition (%)					
Dry Matter DM	97.08 ± 0.06	95.78 ± 0.13	94.93 ± 0.05	93.63 ± 0.05	92.70 ± 0.04
Crude protein, CP	51.57 ± 0.13	50.75 ± 2.57	50.39 ± 0.28	51.23 ± 1.49	50.50 ± 3.15
Crude lipid, CL	14.38 ± 0.64	13.10 ± 0.42	12.93 ± 0.38	13.24 ± 0.46	12.99 ± 0.51
Nitrogen-free extract	21.32 ± 0.34	20.82 ± 1.00	20.64 ± 0.55	19.03 ± 0.67	18.47 ± 1.26
Ash	9.81 ± 0.25	11.11 ± 0.01	10.97 ± 0.00	10.13 ± 0.06	10.74 ± 0.13

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

feeding in the morning) on the rotifer *Brachionus plicatilis* (5 ind/mL) according to Lawrence et al. (2012). Fish were sampled at 21 (larvae), 60 (juvenile, deprived of viscera) and 180 (adult, deprived of viscera, and separating samples from male and female specimens) days post fertilization (dpf), euthanized with a lethal dose of MS222 (1 g/L) (Truzzi et al., 2022) and stored at − 80 °C for further analyses.

2.6. Lipid extraction and fatty acid quantification

Experimental diets and fish (larvae, 10 per tank, juvenile, 5 per tank, adult, 5 per tank and per sex) were analysed for lipid content and FAs composition. Samples were minced and homogenized (homogenizer MZ 4110, DCG Eltronic, Monza, Italy) and freeze-dried (Edwards EF4, Crawley, Sussex, England). Aliquots of 200 mg of each sample (three aliquots per samples) were added with 100 µl of Internal Standard (methyl ester of nonadecanoic acid, 99.6 %, Dr. Ehrenstorfer GmbH, Germany), and: (i) experimental diets were extracted overnight with the Folch method (Folch et al., 1957); (ii) lipid extraction in zebrafish samples were carried out on lyophilized powders following a Microwave-Assisted Extraction (Truzzi et al., 2018a). All lipid extracts were evaporated under laminar flow inert gas (N_2) until constant weight and re-suspended in 0.5 mL of n-heptane. Fatty acid methyl esters (FAMEs) were prepared according to Canonico et al. (2016) using methyl ester of nonadecanoic acid (19:0; Dr. Ehrenstorfer GmbH) as internal standard. FAMEs were determined by an Agilent-6890 Gas-Chromatographic (GC) System (Milano, Italy) coupled to an Agilent-5973 N quadrupole Mass Selective Detector (MS) (Milano, Italy). A CPS ANALITICA CC-wax-MS (30 m × 0.25 mm ID, 0.25 µm film thickness) capillary column was used to separate FAMEs. Instrumental conditions for the studied matrices were set up, according to Truzzi et al. (2017). Data were collected under scan mode for FAMEs identification, and in SIM mode for their quantification. After a solvent delay of 2.0 min, the following fragment ions were recorded: *m/z* 74 and 87 for saturated, *m/z* 74 and 55 for monoenoic FAs, *m/z* 67 and 81 for dienoic FAs, and *m/z* 79 and 81 for other polyunsaturated FAs (Thurnhofer and Vetter, 2006; Zhang et al., 2014).

For each analysed aliquot, at least three runs were performed on the GC-MS. The mass fraction of FAs in mg g^{-1} tissue dry weight (dw) was measured using the response factor method against nonadecanoic acid methyl ester used as internal standard (Truzzi et al., 2018a). The method performances were as those obtained for the determination of FAMEs in insects, in experimental diets of the experiment performed, and in other fish (Truzzi et al., 2018b; Vargas-Abúndez et al., 2019): the linearity was checked up to 320 mg mL^{-1}, and the limit of detection and limit of quantification, calculated as reported by Truzzi et al. (2014)), ranged from 4 mg mL^{-1} to 22 mg mL^{-1} and from 13 mg mL^{-1} to 66 mg mL^{-1}, respectively.

2.7. Statistical analysis

To compare FAs composition between groups, the one-way-ANOVA test, followed by the Multiple Range Test (Daniel and Cross, 2013) was performed after testing the homogeneity of variance with Levene's test. Significant differences were evaluated at the 95 % confidence level. When the ANOVA test gave a p-value equal to 0.0000, in the text it was indicated as $p < 0.0001$. Principal Component Analysis (PCA) was carried out on standardized data; significant components were obtained through the Wold cross-validation procedure (Wold, 1978). ANOVA test and PCA were performed using Statgraphics Plus 19 (2019, Manugistics Inc., Rockville, Maryland, USA).

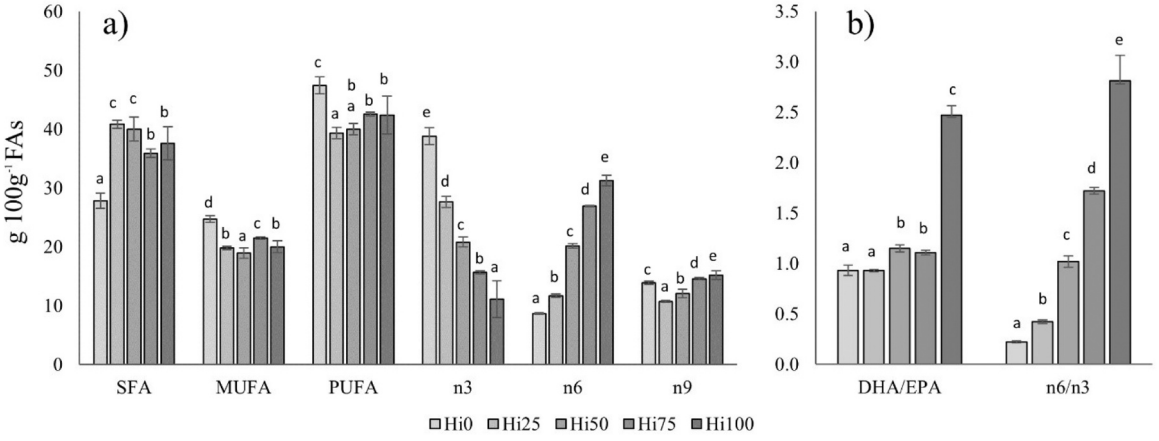


Fig. 1. Fatty acid classes of the experimental diets: a) content of saturated (SFA), monounsaturated (MUFA) and polyunsaturated (PUFA) FA (g 100 g^{-1} FAs), and contribution of omega 3 (n3), omega 6 (n6) and omega 9 (n9) FAs to lipid profile of experimental diets; b) docosahexaenoic (DHA) and eicosapentaenoic (EPA) acids ratio, and n6/n3 ratio. Hi0 (control), Hi25, Hi50, Hi75 and Hi100 diets were characterized by 0, 25, 50, 75 or 100 % inclusion of Hi meal, respectively. Different letters indicate statistically significant differences among experimental groups compared within the same FAs class or the same ratio ($P < 0.05$). Values are presented as mean ± SD ($n = 9$).

3. Results

3.1. Biometry

Data about biometrical parameters of larvae, juvenile and adult female were already reported in Zarantoniello et al. (2020a), Zarantoniello et al. (2020b) and Chemello et al. (2022), respectively. Briefly, SGR % of larvae fed Hi50, Hi75 and Hi100 diets (27.6 ± 0.5, 27.8 ± 0.4, 28.4 ± 0.3 %, respectively) showed significantly higher values ($p < 0.05$) compared to both larvae fed Hi0 (25.4 ± 0.7 %) and Hi25 (25.7 ± 1 %) ones. SGR % of juvenile fed Hi75 (13.0 ± 0.4 %) and Hi100 (13.2 ± 0.4 %) diets was significantly higher ($p < 0.05$) than control group (12.3 ± 0.6 %). Adult female fed Hi0 and Hi25 diets showed significantly higher standard length (25.63 ± 1.9 and 25.9 ± 1.7 mm, respectively) compared to Hi100 group (23.8 ± 1.5 mm), while Hi50 and Hi75 groups were characterized by intermediate values. No significant differences were detected among the experimental adult groups considering wet weight.

3.2. Fatty acid quantification of experimental diets

Results about the FAs classes of Hi-based diets tested in this study were reported in previous papers (Zarantoniello et al., 2020a, 2020b, 2021; Chemello et al., 2022) and showed in Fig. 1. Briefly, a significantly higher content of SFA ($P < 0.05$) and n6 ($P < 0.05$), and a significantly lower content of MUFA ($P < 0.05$), PUFA ($P < 0.05$), and omega3 ($P < 0.05$) with respect to the control diet were detected in Hi-based diets (Fig. 1a). The omega6/omega3 (n6/n3), such as the DHA/EPA, ratios of experimental diets, increased significantly ($P < 0.05$) with the increasing percentage of Hi prepupae meal in the diet (Fig. 1b). Table 2 shows the FAs composition of the experimental diets. In the control diet (Hi0) the most representative FAs are the omega3 eicosapentaenoic (EPA, 20:5n3) and docosahexaenoic (DHA, 22:6n3) acids, followed by 16:0, 18:1n9, 18:2n6, 16:1n7, 14:0 and 18:0. With respect to the control diet (Hi0), diets where fish meal was replaced with Hi meal showed: i) a statistically significant increase ($P < 0.05$) of 12:0; 16:0, and 18:0; ii) a statistically significant increase ($P < 0.05$) of 18:1n9 and 18:3n3 only in Hi75 and Hi100 diets; iii) a statistically significant increase ($P < 0.05$) of 18:2n6, 20:0, and 20:4n6, and a statistically significant decrease ($P < 0.05$) of 20:5n3, 22:6n3, 14:0, 16:1n7, 18:1n7, with increasing the percentage of Hi meal in the diet.

3.3. Fatty acid quantification in Danio rerio

The dry weight (dw) of zebrafish specimens in the different life stages was 15.7 ± 0.8 % for larvae, 22.9 ± 0.9 % for juveniles, 30.5 ± 1.6 % for adult males, and 33.1 ± 0.8 % for adult females. No statistically significant differences were evidenced between groups inside the same life stage. Total lipid content (g 100 g^{-1} dw) increased passing from larvae (14 ± 1 %), to juveniles (20 ± 1 %), adult males (24 ± 3 %), and adult females (29 ± 3 %). Statistically significant differences were evidenced between larvae, juvenile and adult ($p < 0.0001$, no differences between adult female and adult male), but not between groups of specimens inside the same life stage fed different diets.

Fig. S1 shows an example of chromatogram of FAs profile obtained from an adult male zebrafish. Table 3 reports the FA class

Table 2
Fatty Acid composition (g 100 g^{-1} of total FAs) of experimental diets in which fish meal was replaced for 0, 25 %, 50 %, 75 % and 100 % with Hi meal (Hi0, Hi25, Hi50, Hi75 and Hi100, respectively).

	Hi0	Hi25	Hi50	Hi75	Hi100
10:0	<dl	0.45 ± 0.01	0.47 ± 0.02	0.42 ± 0.03	0.50 ± 0.04
12:0	0.20 ± 0.01^{a}	7.3 ± 0.2^{c}	6.4 ± 0.2^{b}	6.0 ± 0.4^{b}	6.2 ± 0.3^{b}
14:0	6.9 ± 0.3^{e}	5.7 ± 0.1^{d}	4.0 ± 0.1^{c}	3.0 ± 0.2^{b}	2.0 ± 0.2^{a}
15:0	0.67 ± 0.04	0.52 ± 0.01	0.44 ± 0.02	0.37 ± 0.01	0.29 ± 0.01
16:0	15 ± 1^{a}	16 ± 1^{ab}	18 ± 2^{b}	17 ± 1^{b}	17 ± 1^{b}
16:1n9	0.37 ± 0.05	0.23 ± 0.04	0.27 ± 0.03	0.35 ± 0.05	0.23 ± 0.01
16:1n7	8.0 ± 0.3^{d}	6.9 ± 0.2^{c}	5.1 ± 0.2^{b}	4.8 ± 0.3^{b}	3.2 ± 0.1^{a}
17:0	0.54 ± 0.03	0.38 ± 0.01	0.35 ± 0.03	0.30 ± 0.01	0.29 ± 0.01
17:1n7	0.44 ± 0.03	0.39 ± 0.03	0.52 ± 0.05	0.73 ± 0.06	0.71 ± 0.01
18:0	4.2 ± 0.4^{a}	9.4 ± 0.7^{b}	8.9 ± 0.5^{b}	8.3 ± 0.6^{b}	8.9 ± 0.6^{b}
18:1n9	11.6 ± 0.7^{a}	10.5 ± 0.5^{a}	12.5 ± 0.7^{a}	14.0 ± 0.6^{b}	14.8 ± 0.7^{b}
18:1n7	2.5 ± 0.1^{d}	1.8 ± 0.1^{c}	1.5 ± 0.2^{bc}	1.4 ± 0.1^{b}	1.0 ± 0.1^{a}
18:2n6	8.0 ± 0.5^{a}	10.5 ± 0.6^{b}	17.5 ± 1.2^{c}	25.9 ± 1.5^{d}	28.9 ± 1.8^{d}
18:3n6	0.50 ± 0.03	0.21 ± 0.03	<dl	<dl	<dl
18:3n3	1.9 ± 0.2^{a}	1.6 ± 0.2^{a}	1.6 ± 0.2^{a}	2.7 ± 0.2^{b}	3.0 ± 0.3^{b}
20:0	0.37 ± 0.03^{a}	0.71 ± 0.01^{b}	1.1 ± 0.1^{c}	1.0 ± 0.1^{c}	2.6 ± 0.2^{d}
20:4n6	0.19 ± 0.02^{a}	1.0 ± 0.2^{b}	1.2 ± 0.1^{b}	1.1 ± 0.1^{b}	2.3 ± 0.1^{c}
20:3n3	<dl	1.2 ± 0.1^{a}	1.0 ± 0.2^{a}	1.3 ± 0.3^{a}	2.2 ± 0.3^{b}
20:5n3	19.1 ± 0.3^{e}	12.9 ± 0.1^{d}	8.7 ± 0.3^{c}	5.5 ± 0.1^{b}	1.7 ± 0.1^{a}
22:1n9	1.2 ± 0.1	0.21 ± 0.04	0.21 ± 0.01	0.26 ± 0.01	0.15 ± 0.01
22:6n3	17.8 ± 0.3^{e}	12.0 ± 0.1^{d}	10.0 ± 0.1^{c}	6.1 ± 0.1^{b}	4.2 ± 0.3^{a}
24:1n9	0.78 ± 0.04	0.50 ± 0.01	<dl	<dl	<dl

dl: detection limit. Means within rows of experimental diets bearing different letters are significantly different ($P < 0.05$). FAs < 1 % were excluded from any statistical analyses because their concentrations were close to the dl.

Table 3
Content of fatty acids classes (mg g^{-1} dw) of zebrafish specimens fed experimental diets.

	Hi0	Hi25	Hi50	Hi75	HI100
SFA					
larvae	37 ± 1^{a}	38 ± 1^{a}	40 ± 1^{a}	43 ± 1^{a}	45 ± 1^{a}
juvenile	64 ± 2^{b}	73 ± 2^{b}	77 ± 3^{b}	81 ± 3^{b}	90 ± 1^{c}
Adult male	68 ± 3^{b}	77 ± 3^{b}	86 ± 4^{c}	86 ± 5^{b}	85 ± 4^{b}
Adult female	66 ± 5^{b}	72 ± 3^{b}	75 ± 5^{b}	87 ± 5^{b}	84 ± 4^{b}
MUFA					
larvae	42 ± 1^{a}	43 ± 1^{a}	41 ± 2^{a}	39 ± 1^{a}	39 ± 1^{a}
juvenile	72 ± 3^{b}	70 ± 1^{b}	69 ± 3^{b}	67 ± 3^{b}	69 ± 1^{b}
Adult male	77 ± 3^{b}	80 ± 2^{c}	82 ± 5^{c}	85 ± 3^{c}	91 ± 4^{c}
Adult female	75 ± 3^{b}	77 ± 3^{c}	78 ± 4^{c}	82 ± 3^{c}	91 ± 4^{c}
PUFA					
larvae	56 ± 2^{a}	55 ± 1^{a}	51 ± 1^{a}	50 ± 2^{a}	48 ± 1^{a}
juvenile	109 ± 3^{b}	98 ± 4^{b}	84 ± 3^{b}	78 ± 2^{b}	77 ± 2^{b}
Adult male	118 ± 4^{c}	113 ± 4^{c}	99 ± 3^{c}	95 ± 3^{c}	85 ± 4^{c}
Adult female	132 ± 3^{d}	125 ± 3^{d}	105 ± 3^{d}	100 ± 3^{c}	82 ± 2^{c}
n3					
larvae	38 ± 1^{a}	36 ± 1^{a}	31 ± 1^{a}	26 ± 1^{a}	24 ± 1^{a}
juvenile	77 ± 3^{b}	65 ± 3^{b}	48 ± 3^{b}	35 ± 2^{b}	30 ± 1^{b}
Adult male	82 ± 4^{b}	75 ± 4^{c}	58 ± 2^{c}	44 ± 3^{c}	33 ± 3^{b}
Adult female	96 ± 3^{c}	89 ± 3^{d}	62 ± 2^{d}	51 ± 2^{d}	32 ± 2^{b}
n6					
larvae	17.7 ± 0.8^{a}	19.3 ± 0.5^{a}	19.8 ± 0.5^{a}	23.7 ± 0.5^{a}	24.2 ± 0.9^{a}
juvenile	31.8 ± 0.9^{b}	33.4 ± 0.6^{b}	36 ± 1^{b}	43 ± 1^{b}	47 ± 1^{b}
Adult male	35 ± 2^{c}	38 ± 2^{c}	40 ± 2^{c}	50 ± 1^{c}	52 ± 2^{c}
Adult female	35 ± 2^{c}	36 ± 2^{c}	42 ± 1^{c}	49 ± 3^{c}	49 ± 2^{bc}
n9					
larvae	23 ± 1^{a}	24 ± 1^{a}	24 ± 1^{a}	23 ± 1^{a}	23 ± 1^{a}
juvenile	42 ± 2^{b}	40 ± 1^{b}	41 ± 2^{b}	39 ± 2^{b}	40 ± 1^{b}
Adult male	48 ± 2^{c}	50 ± 2^{c}	55 ± 5^{c}	60 ± 4^{c}	68 ± 4^{c}
Adult female	48 ± 2^{c}	52 ± 3^{c}	55 ± 4^{c}	60 ± 3^{c}	69 ± 4^{c}
Total FAs					
larvae	136 ± 5^{a}	137 ± 5^{a}	132 ± 6^{a}	132 ± 5^{a}	132 ± 6^{a}
juvenile	244 ± 10^{b}	241 ± 8^{b}	229 ± 8^{b}	226 ± 10^{b}	236 ± 7^{b}
Adult male	263 ± 5^{c}	270 ± 6^{c}	267 ± 5^{c}	266 ± 6^{c}	262 ± 6^{c}
Adult female	273 ± 10^{c}	274 ± 8^{c}	259 ± 8^{c}	269 ± 7^{c}	257 ± 10^{c}

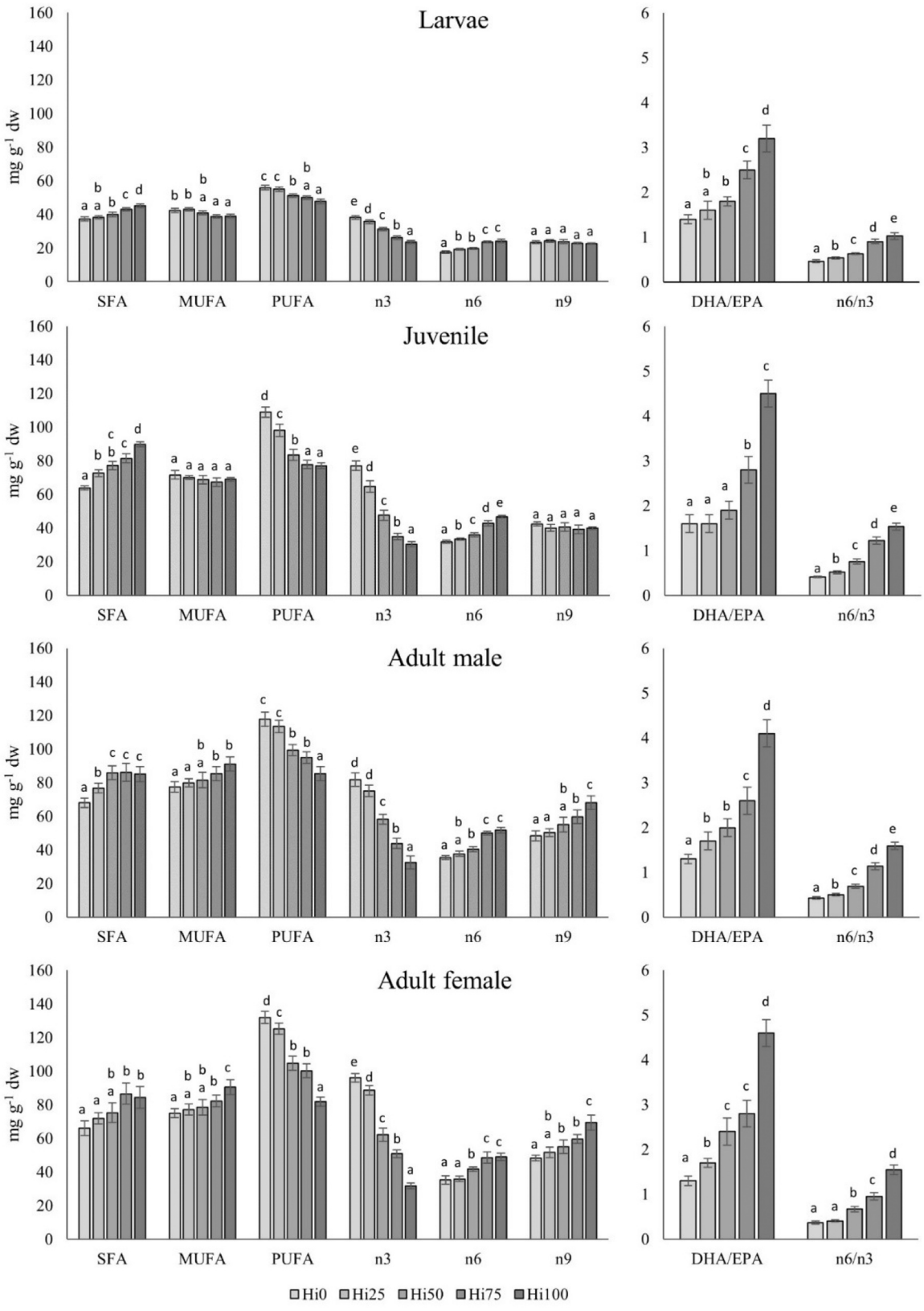
Content of saturated (SFA), monounsaturated (MUFA) and polyunsaturated (PUFA) FAs, omega 3 (n3), omega 6 (n6) and omega 9 (n9) FAs, in zebrafish specimen fed diets including 0, 25 %, 50 %, 75 % and 100 % of Hi meal in substitution of fish meal (Hi, Hi25, Hi50, Hi75 and Hi100, respectively). Different letters in column indicate statistically significant differences among different life stages of zebrafish fed the same diet for each FA class ($p < 0.05$). Values are presented as mean ± SD ($n = 9$).

content (mg g^{-1} dw) for zebrafish larvae, juveniles, adult males and adult females fed with the experimental diets and shows the statistical analysis applied, for each FA class, to different life stages of zebrafish fed the same diet Fig. 2 shows graphically the quantification (mg g^{-1} dw) of FAs classes for each life stage considered and reports the statistical analysis applied, for each FA class, to specimens belonging from the same life stage and fed with different experimental diets. Fig. 3 reports the contour plot, that showed the influence of both life stage (larvae, juvenile and adult) and experimental diets on the content of FAs classes (SFA, MUFA and PUFA) and on the DHA/EPA ratio.

3.3.1. Fatty acids classes

The total FAs content of experimental groups belonging to the same life stage did not vary with dietary variation ($P > 0.05$) (Table 3). Zebrafish larvae showed an overall FAs content (134 ± 2 mg g^{-1} dw) significantly lower ($P < 0.01$) than the other life stages (235 ± 8, 266 ± 3 and 266 ± 8 mg g^{-1} dw, for juveniles, adult males and adult females, respectively), and in turn juveniles showed a total FAs content significantly lower than both male and female adult specimens ($P < 0.05$). Generally, within specimens fed with the same diet, larvae showed a statistically significant lower content of all considered FAs classes with respect to the other life stages ($P < 0.05$), (Table 3), whereas juveniles showed a statistically significant lower content of MUFA, PUFA, omega3, omega6 and omega9 than adult specimens ($P < 0.05$) (Table 3). Moreover, comparing specimens fed with the same diet, all experimental groups of adult males (except specimen fed with Hi100 diet) showed a statistically significant lower content of n3 with respect to adult females ($P < 0.05$) (Table 3), leading to a lower PUFA content.

SFA generally increased significantly in fish with increasing the level of Hi meal in the diet in all life stages considered, as observed in Figs. 2 and 3 (see also Table 3 for data). MUFA showed a different behaviour depending on the life stage. In larvae, MUFA decreased significantly ($P < 0.05$) in fish fed with Hi75 and Hi100 diets with respect to fish fed with the control diet. In juveniles no statistically significant differences ($P > 0.05$) were evidenced in MUFA content between specimen fed with the different diets. In adult specimens, both males and females, MUFA increased significantly in fish fed with Hi75 and Hi100 diets with respect to fish fed with the control diet ($P < 0.05$). PUFA are the most represented FAs class in specimen fed with the control diet for all life stages. PUFA generally



(caption on next page)

Fig. 2. Content of saturated (SFA), monounsaturated (MUFA) and polyunsaturated (PUFA) FAs (mg g^{-1} dw), omega 3 (n3), omega 6 (n6) and omega 9 (n9) FAs, and docosahexaenoic (DHA) and eicosapentaenoic (EPA) acids ratio and n6/n3 ratio of zebrafish specimen fed diets including 0, 25 %, 50 %, 75 % and 100 % of Hi meal (Hi, Hi25, Hi50, Hi75 and Hi100, respectively). Different letters indicate statistically significant differences among experimental groups compared within the same FAs class or ratio ($p < 0.05$). Values are presented as mean ± SD ($n = 9$).

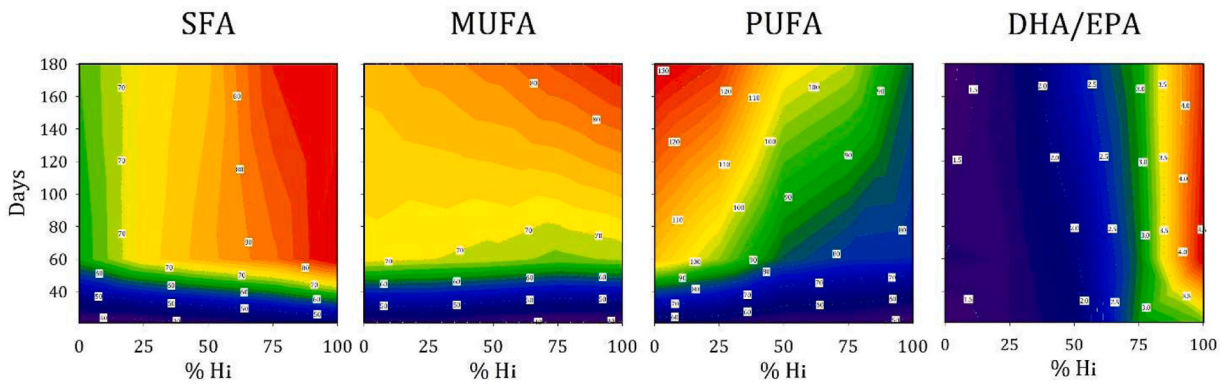


Fig. 3. Contour plot. Saturated (SFA), monounsaturated (MUFA) and polyunsaturated (PUFA) fatty acids and docosahexaenoic (DHA) and eicosapentaenoic (EPA) acids ratio.

decreased significantly with the increasing level of Hi meal in the diet in all considered life stage, as observed also in Fig. 3. Moreover, with the increasing level of Hi meal in the diet, we observed (Fig. 2, see Table 3 for data): i) a statistically significant decrease of omega3 in all life stages ($P < 0.05$); ii) a statistically significant increase of omega6 in all life stages ($P < 0.05$); iii) a statistically significant increase of omega9 for adult males and for adult females ($P < 0.05$). No statistically significant differences in n9 content were evidenced between groups fed with different diets neither in larvae ($P > 0.05$), nor in juveniles ($P > 0.05$). The behaviour of omega3 and omega6 determined a statistically significant increase of the n6/n3 ratio in all life stages with increasing the level of Hi meal in the diet (Fig. 2 and Tables S1-S4) ($P < 0.05$).

3.3.2. Quantification of individual fatty acids

Tables S1-S4 show the FAs composition (as mg g^{-1} dw) of zebrafish larvae, juveniles, adult males and females, respectively, fed with the experimental diets (Hi0, Hi25, Hi50, Hi75 and Hi100). A total of 29 FAs were found in zebrafish specimens. Fig. 4 shows the trend of the main FAs most sensitive to the diet, for each FAs class and for each life stage, in relation to the administered experimental diets. Between SFA, the most represented in fish fed with control diet Hi0 was, for all life stages, palmitic acid (16:0, ~20 mg g^{-1} in larvae, 38–43 mg g^{-1} for the other life stages), followed by stearic (18:0, 8–11 mg g^{-1} for all life stages), myristic (14:0, ~5.5 mg g^{-1} in larvae, ~11 mg g^{-1} in the other life stages), 17:0 (~2 mg g^{-1} for all life stages), and 15:0 (~1 mg g^{-1} in larvae, ~2 mg g^{-1} in the other life stages) acids (Tables S1-S4). Other minor SFA were 10:0, 12:0, 13:0, 20:0 and 22:0, with a content ≤ 1 mg g^{-1} or below the detection limit for all groups. Lauric acid (12:0) showed, in all life stages, a statistically significant increase ($P < 0.0001$) with increasing the level of Hi meal in the diet, passing from a content ≤ 1 mg g^{-1} in all group of fish fed Hi0 diet, to a content, for specimens fed with Hi100 diet, of: ~7 mg g^{-1} in larvae, ~19.5 mg g^{-1} in juveniles, ~14.7 mg g^{-1} in adult males, ~13.4 mg g^{-1} in adult females (Tables S1-S4 and Fig. 4). Palmitic acid (16:0) increased significantly ($P < 0.05$) only in juvenile fed Hi100 diet, with respect to specimens fed control diet. Stearic acid (18:0) increased significantly ($P < 0.05$) only in adult female fed Hi75 and Hi100 diet, with respect to specimens fed control diet.

As regards MUFA, the most represented in fish fed with control diet Hi0 was, for all life stages, oleic acid (18:1n9, ~20 mg g^{-1} in larvae, ~36 mg g^{-1} in juveniles, ~41 mg g^{-1} in adult specimens), followed by palmitoleic acid 16:1n7 (~12 mg g^{-1} in larvae, 18–20 mg g^{-1} in other life stages), 18:1n7 (7–8 mg g^{-1} for all life stages), and other minor FAs, with a content of few mg g^{-1} or below the detection limit, such as 14:1n5, 16:1n9, 20:1n9, 22:1n9, 24:1n9, and 17:1n7 (Tables S1-S4). With respect to fish fed the control diet (Hi0), 18:1n9 showed a statistically significant increase only in adult specimens (both males and females) fed diets included of Hi meal level > 50 % ($P < 0.05$). Moreover, adult males and females fed Hi100 showed a statistically significant increase of 18:1n9 content also with respect to fish fed with the other dietary treatments ($P < 0.05$) (Fig. 4). Other MUFA such as 16:1n7, 18:1n7, 20:1n9 and 22:1n9 showed a statistically significant general decrease ($P < 0.05$) in fish for all life stages with increasing the inclusion level of Hi meal in the diet (Fig. 4 and Tables S1-S4).

Between PUFA, the most represented was, for control specimens of all life stages, docosahexaenoic acid (DHA, 22:6n3, ~19 mg g^{-1} in larvae, 44 mg g^{-1} in juveniles, ~43 mg g^{-1} in adult males, 51 mg g^{-1} in adult females), followed by eicosapentaenoic acid (EPA, 20:5n3, ~14 mg g^{-1} in larvae, 27 mg g^{-1} in juveniles, ~33 mg g^{-1} in adult males, 39 mg g^{-1} in adult females), linoleic acid (18:2n6, ~13 mg g^{-1} in larvae, ~26 mg g^{-1} in juvenile, ~30 mg g^{-1} in adult specimen), α-linolenic acid (18:3n3, ~5 mg g^{-1} for all life stages), and arachidonic acid (20:4n6, 3–4 mg g^{-1} for all life stages) (Tables S1-S4). Other minor PUFA with a content ≤ 1 mg g^{-1} or below the detection limit for all groups were 16:2n7, 18:3n6, 20:2n6, 20:3n6, 20:3n3. With increasing the inclusion level of Hi meal in the diet,

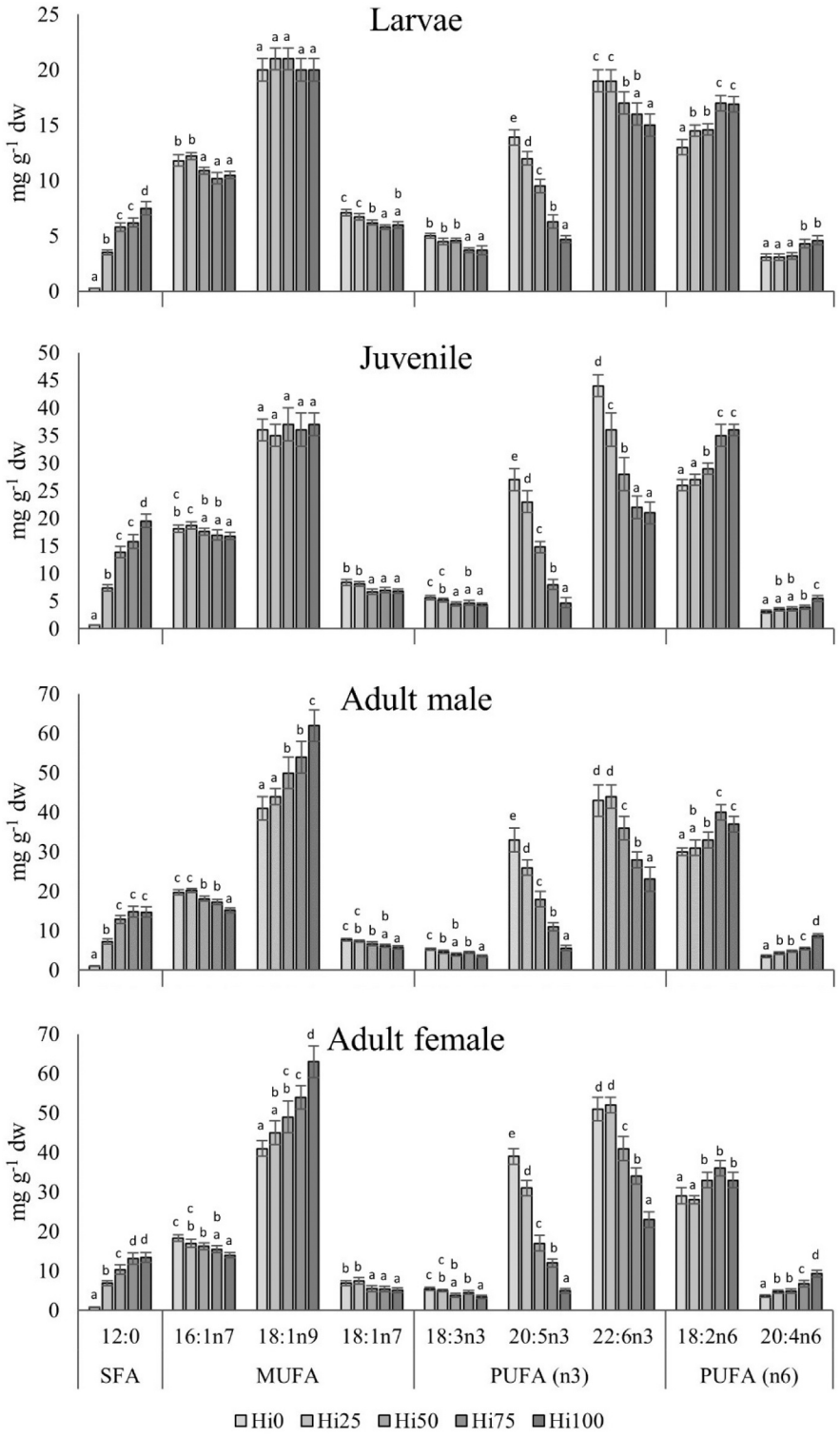


Fig. 4. Trend, for each FAs class, of the main FAs sensitive to the experimental diets, for each life stage of zebrafish fed diets including 0, 25 %, 50 %, 75 % and 100 % of Hi meal (Hi, Hi25, Hi50, Hi75 and Hi100). Different letters indicate statistically significant differences among experimental groups compared within the same FA ($p < 0.05$). Values are presented as mean ± SD ($n = 9$).

we observed in zebrafish, for all life stages, a statistically significant decrease of the most important omega3, 18:3n3 ($P < 0.05$), 22:6n3 ($P < 0.05$) and 20:5n3 ($P < 0.05$). Passing from fish fed with Hi0 diet to fish fed with Hi100 diet, 22:6n3 showed a decrease of ~20 % for larvae and ~50 % for the other life stages, whereas 20:5n3 decreased of ~65 % for larvae, and ~85 % for juveniles and adult specimens. The greater decrease percentage of EPA with respect to DHA for all life stages determined an increase of the DHA/EPA ratio in fish (Table S1-S4) with increasing the inclusion level of Hi meal in the diet ($P < 0.05$), as graphically observed also in Fig. 3. Conversely, increasing the inclusion level of Hi meal in the diet, we observed, for all life stage, a statistically significant increase

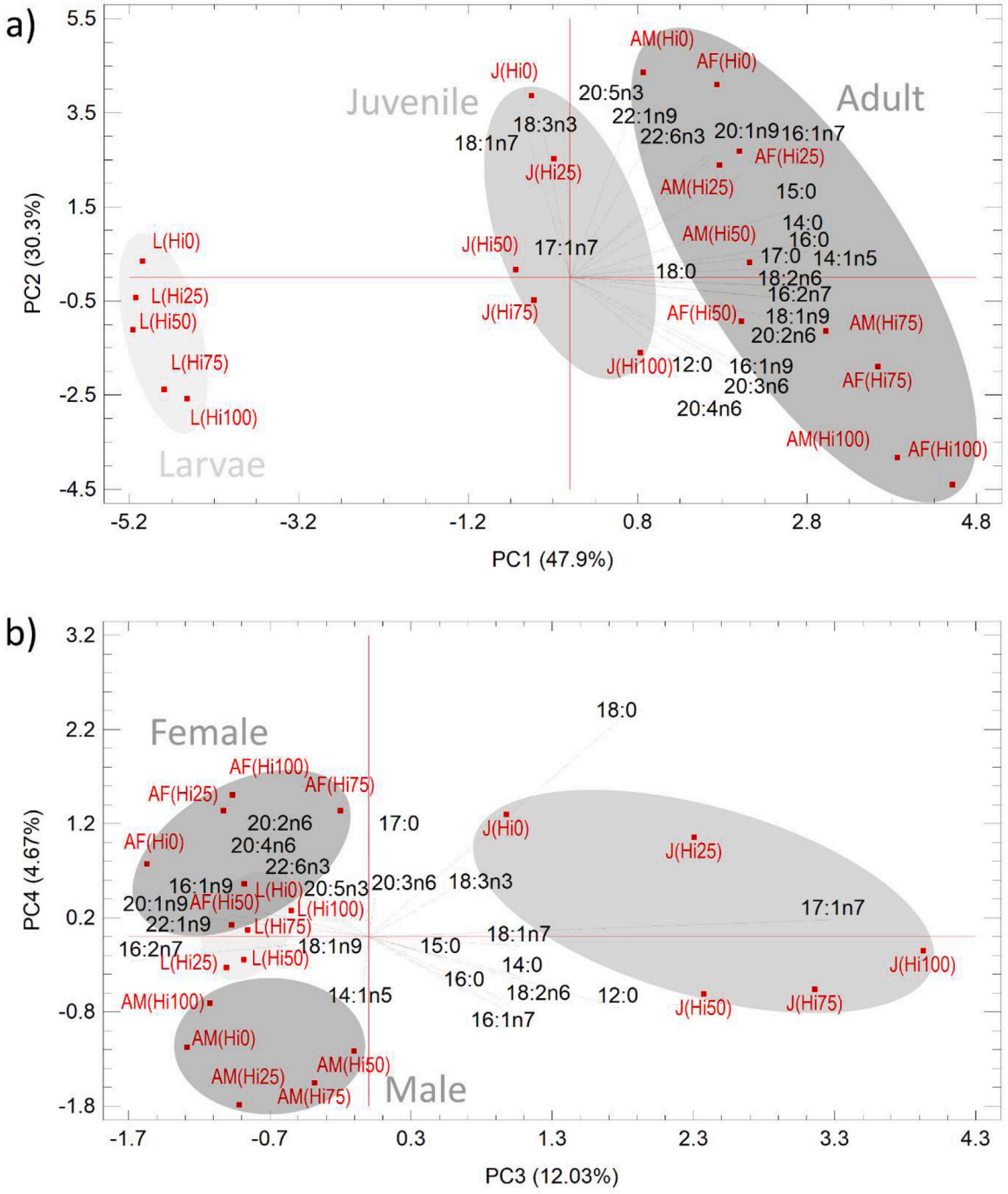


Fig. 5. Principal Component Analysis: 6a, 2D Biplot of PC1 (first Principal Component) versus PC2 (second Principal Component); 6b, 2D Biplot of PC3 (third Principal Component) versus PC4 (fourth Principal Component). Zebrafish specimens (L, larvae; J, juvenile; AM, adult male; AF, adult female) fed diets including 0, 25 %, 50 %, 75 % and 100 % of Hi meal (Hi, Hi25, Hi50, Hi75 and Hi100).

($P < 0.05$) of various omega6, such as 18:2n6, 20:4n6 (Tables S1-S4 and Fig. 4), and 20:2n6, 20:3n6 (Tables S1-S4). Comparing FA content between different life stages of specimen fed control diet (Tables S1-S4), we observed that larvae showed a statistically significant lower content ($P < 0.05$) of all FAs, except 18:3n3 and 20:4n6, with respect to the other life stages. Juveniles showed a statistically significant lower content ($P < 0.05$) of 18:1n9, 20:1n9, 22:1n9, 20:5n3, 18:2n6, and 16:2n7 (<dl) with respect to both male and female adult specimens, and of 22:6n3 and 20:3n6 with respect to adult females. Finally, adult males showed a statistically significant lower content ($P < 0.05$) of 22:6n3 (DHA), 20:5n3 (EPA), and 20:3n6 with respect to adult females.

3.4. Principal component analysis

To better understand the relationship between the inclusion of Hi meal in the diet and the content and composition of FAs in the different life stages of zebrafish, a multivariate analysis (Principal Component Analysis, PCA) was performed to reduce the dimensionality of the data set to few components that summarize the information contained in the overall data set. FAs with a content lower than LOQ were excluded from the data matrix (10:0, 13:0, 20:0, 22:0, 18:3n6, 20:3n3, 24:1n9). By applying PCA to the data set (16 observations, 22 variables), it was possible to extract 4 significant cross-validated principal components, that accounted for 94.9 % of the variability (Table S5). On examining the loading matrix (Table S5) and the graphical distribution of analysed groups on the reported biplot (showing *loadings* and *scores* plots simultaneously) of PC1 vs PC2 (Fig. 5a), specimens were divided based on their life stage and on their diet composition. PC1, that explained 47.9 % of the variance, was associated to the overall content of FAs, most of them having positive loadings on the first PC1. Larvae (negative scores) showed the lowest content of FAs, and adult specimens (positive scores) showed the highest content of FAs. Juveniles had scores next to 0 value, between larvae and adult groups. PC2, that explained 30.3 % of the variance, was dominated by the diet, with experimental groups of fish passing from positive to negative scores with increasing the inclusion of Hi prepupae in their diet. In particular, specimens fed with the control diet Hi0 and the Hi25 diet (positive scores) were characterized by a higher content of omega3 and omega9 (highest factor loadings on PC2) with respect to specimens fed with Hi50, HI75 and Hi100 diet (negative scores), which in turn are characterized by a higher content of omega6 (lowest factor loadings on PC2) (Fig. 5a and Table 3). PC3 (12.0 %) and PC4 (4.7 %) were associated to the different FAs composition between specimens of different life stages (Fig. 5b). Juvenile (positive score on PC3) is the only life stage with a content of 17:1n7 higher than the detection limit, and it showed a higher content of 18:0 and 12:0 with respect to larvae and adult specimens (negative scores on PC3). PC4 was dominated by the different FAs composition between adult females (positive scores) and adult males (negative scores). In particular, females, with respect to males, showed: i) a higher content of 17:0 and 18:0 and a lower content of 12:0, 14:0, and 16:0; ii) a lower content of MUFA such as 14:1n5 and 16:1n7; iii) a lower content of 18:2n6 and a higher content of 20:2n6, 20:3n6 and 20:4n6; iv) a higher content of 20:5n3 and 22:6n3 (Table S5 and Fig 6b).

4. Discussion

The aim of this research was to study the profile and the content of FAs in the experimental model *Danio rerio*, in relation to the FAs composition of the feed and to the fish life stage. Previous studies in which *Hermetia illucens* (Hi), fed on coffee roasted by-product added with 10 % *Schyzochytrium* sp., was processed into meal and used to replace FM (in the percentage of 0, 25 %, 50 %, 75 % and 100 %) in five experimental diets to be tested in a feeding trial using zebrafish (*Danio rerio*) as experimental model (Zarantoniello et al., 2020a, 2020b, 2021; Chemello et al., 2022), demonstrated a modification of FAs profile, expressed as percentage of each FA vs total FAs, in relation to the diet in all life stage (larvae, juveniles and adult females). Moreover, they demonstrated that: i) no alteration of diet ingestion among all the experimental groups were recorded; ii) the inclusion of Hi meal in the diet did not affect neither growth performance (except for body length in juveniles) nor the somatic indexes; iii) higher replacement levels (Hi75 and Hi100) affected liver histology and induced a general increase in lipid accumulation (hepatic steatosis) and stress response; iii) in adult females, Hi75 and Hi100 diet affected oocyte maturation stages, spawning and hatching success. Specifically, data on immune response, lipid metabolism, chitinolytic activity and stress response as well as gut and liver welfare of zebrafish specimens analyzed in this study are detailed in Zarantoniello et al. (2018, 2020a, 2020b, 2021). However, since the physiological responses are part of other papers, they are not directly presented in the present one.

Results on the variation of FAs content in mg g^{-1} dw in relation to feed composition in larvae, juveniles and adult females are mostly consistent, within the experimental error, with previous data, where FAs content was expressed in percentage of total FAs (Zarantoniello et al., 2020a, 2020b; Chemello et al., 2022). Data about FAs content as percentage of total FAs in adult males were not published. In this study, the PCA evidenced as FAs composition of *Danio rerio* was influenced by both life stage and diet.

The inclusion of insect meal in the diet of the zebrafish experimental model (even only 25 %), lead to a significant increase in the diet SFA content, as reported in the literature (Oonincx et al., 2015; Zarantoniello et al., 2020b). Among SFA, the most abundant in the diet was 16:0 (palmitic acid), but the most significant change in relation to the increase in the percentage of inclusion of Hi in the diet was that of 12:0 (lauric acid), which increased up to 30 times from control diets to diet Hi100. The abundance of SFA in feed was reflected in a significant increase in SFA in all life stages of zebrafish: the most abundant SFA was 16:0, followed by 18:9, 14:0 and 12:0. Similarly to the diet, the most substantial change occurred in lauric acid, which increased up to about 30 times in larvae and juveniles, up to 15 times in adult males and up to 20 times in adult females. Lauric acid is known to have antibacterial and antiviral activity, and literature data highlighted the possibility that it could contribute to a reduction in the use of antibiotics in aquaculture (Gasco et al., 2018). In addition, this FA can improve intestinal health and prevent inflammatory processes (Aleström et al., 2006; Cardinaletti et al., 2019; Randazzo et al., 2021a, 2021b). A possible drawback related to the insect's dietary inclusion could be related to the presence of chitin (derived from their exoskeleton) that, at certain concentration, could reduce the absorption of nutrients with serious

repercussions on the growth rate of the animal (Dumas et al., 2018; Ratti et al., 2023). However, in this experiment, the biometric data of zebrafish, relating to length and weight (Zarantoniello et al., 2020a, 2020b; Chemello et al., 2022), showed that, with the increasing dietary Hi levels: i) larvae and juveniles showed a significantly higher growth rate than fish fed control diet; ii) adult males showed a significant weight gain, while females showed no statistically significant differences with respect to fish fed control diet. These results demonstrated that the presence of chitin in the diets did not adversely affect the growth rate of *Danio rerio* specimens. The results obtained could be explained by the increased availability of medium chain SFA and, in particular, of lauric acid; in fact, it is known that medium chain FAs (C8-C12) are absorbed, digested and oxidized quickly, thus representing a readily available source of energy (Dayrit, 2015).

In all life stages of zebrafish specimens, the most abundant MUFA was 18:1n9, followed by 16:1n7 and 18:1n7. The significant decrease in MUFA observed in the diet as the percentage of inclusion of Hi increased, is reflected in a decrease of MUFA only in larvae, whereas in juveniles MUFA remained unchanged, and in adults, on the contrary, MUFA increased. The MUFA increase in adult specimens is exclusively due to a significant increase in 18:1n9 with increasing the percentage inclusion of Hi in the diet. These different results between life stages denotes a different response of metabolic pathways of FAs to the feed. It is well known that dietary SFA (i.e, 16:0) and MUFA (i.e., 18:1n9 and 20:1n9) are readily catabolized by mitochondrial β-oxidation to generate metabolic energy in fish. If provided in large amounts, they can be accumulated as a reserve of high-energetic molecules (Turchini et al., 2022). The highlighted differences in MUFA content in fish can be possibly related to the well-known different growth and lipid accumulation rates of larval, juvenile and adult fish (Ju et al., 1997; Zarantoniello et al., 2020b). As reported by previous studies on zebrafish, fish fed Hi-based diets showed better growth performances with respect to a control diet during the larval phase, suggesting the potential role of both SFA and MUFA in provide energy substrates to sustain growth during larval development (Zarantoniello et al., 2018, 2020b). The high catabolism rate can explain the scarce accumulation of MUFA in larval tissues which profile reflects the dietary one. Differently, in adults, due to the lower growth-related metabolic requirements compared to larval phase, high amounts of dietary SFA and MUFA led to an accumulation (causing hepatic steatosis when high levels are reached). The increasing dietary SFA content with the increasing dietary inclusion level of Hi could provide sufficient energy substrates in adult zebrafish leading to a progressive accumulation of MUFA from Hi0 to Hi100, despite the reduction of this FAs in the diets. In feed, a significant decrease of PUFA as the inclusion of Hi meal in the diet increases, was substantially linked to the decrease of two important omega3: EPA (20:5n3, −90 % from Hi0 to Hi100) and DHA (22:6n3, −75 % from Hi0 to Hi100). In zebrafish specimens of all life stage considered, the most abundant PUFA was DHA, followed by EPA, 18:2n6 and 18:3n3. The increase in the dietary Hi inclusion reflected a significant reduction of these essential FAs in all the zebrafish life stages, as observed for the feed. It is noteworthy that the differences in terms of PUFA among groups were more contained within the larvae compared the other life stages: PUFA decreased of about 15 % in larvae, 28 % in juvenile and adult males, and about 37 % in adult females from Hi0 to Hi100 group. These results suggest that the dietary inclusion of Hi prepupae meal can meet the FAs requirement in the short term, but prolonged administration of high Hi inclusion levels could lead to a PUFA deficiency in the body. The highest percentage drop in adult females may be linked to a need to use PUFA for reproduction. As highlighted by PCA, females showed a different FAs profile with respect to male. In particular, the females' FA composition, with respect to male, showed: i) among SFA, a higher content of 17:0 and 18:0 and a lower content of 12:0, 14:0, and 16:0; ii) among MUFA, a lower content of 14:1n5 and 16:1n7; iii) about PUFA, a lower content of 18:2n6 and a higher content of its derivatives, such as 20:3n6 and 20:4n6. In addition, females contained more omega3, such as EPA and DHA, than males. This different FAs profile could be justified by the needs of physiological reproductive processes, as demonstrated by different studies on reproductive performances of zebrafish females fed Hi-based diets (Randazzo et al., 2020; Chemello et al., 2022).

The decrease of the PUFA percentage in response to the increase of Hi prepupae meal in the diets is tied to a substantial decrease of EPA and DHA. In particular, passing from fish fed on the Hi0 diet to fish fed on the Hi100 diet, we observed, for EPA and DHA, respectively, a decrease of 65 % and 20 % per larvae, and 85 % and 50 % for juveniles and adults. Again, it can be noted that, although there is a progressive decrease in these two important FAs, the differences in the composition of the various groups tested are less marked than in feed, highlighting the ability of zebrafish, as a freshwater fish, to activate long chain PUFA synthesis pathways from shorter chain precursors in order to compensate at least partially for the strong reduction of these important FAs in diets (Tocher, 2010). Similar studies that investigated the expression of genes (*elovl2*, *elovl5* and *fads2*) codifying for elongases and desaturases necessary for the synthesis of FAs confirm this hypothesis (Zarantoniello et al., 2018). However, these deficiencies may place restrictions on the inclusion of high proportions of insects in the diet of marine fish that are unable to make these conversions (Holt and Yandell, 2011).

Although the need for essential FAs varies between different species and in relation to the ontogenetic cycle of the animal, the need for omega3-PUFA is not only related to the absolute quantity with which these FAs are supplied, but also the relative proportions between DHA and EPA. In particular, the need for essential FAs is inversely proportional to the DHA/EPA ratio; this is related both to the conversion rate of EPA to DHA, and to the role played by DHA in retinal and cerebral development (Bell et al., 1997; Tocher, 2010; Bruni et al., 2020). Based on the FA composition present in the eggs of many marine fish species, the DHA/EPA ratio considered optimal for the first larval feeding is about 2 (Holt and Yandell, 2011). The data obtained in this paper showed that the dietary inclusion of Hi prepupae meal leads to a significant increase in this ratio, which reaches a value close to the optimal value in feed (up to a maximum of 2.4 $\pm$ 0.2 for Hi100), and grows in all life stages of zebrafish up to a maximum value in fish fed with the Hi100 diet of 3.2 $\pm$ 0.3 in larvae, 4.5 $\pm$ 0.3 in juveniles, 4.1 $\pm$ 0.3 in adult males, and 4.6 $\pm$ 0.3 in adult females. The Hi50 diet allows fish of any life stage to reach the optimal value of about 2.

The progressive Hi prepupae meal dietary inclusion also led to a significant increase ($P < 0.05$) of arachidonic acid (20:4n6), in larvae, juveniles and adults, because of the progressive increase in the essential FA linoleic acid (18:2n6), that promoted an increase of the n6 synthesis.

Similarly, to what has been said for DHA and EPA, it is now widely recognized that in order to achieve an optimal state of health it is necessary to pay attention to the n6/n3 ratio rather than to the absolute quantity of the single FA. With a view to the use of insects in aquaculture, the n6/n3 ratio is also important for humans. In today's western diet this ratio is about 20:1 or more (Zárate et al., 2017), while the optimal ratio should be maximum 5:1 (Patel et al., 2022), being effective in slowing cancer progression (Berquin et al., 2008). Studies suggest that increased intake of n6 may lead to breast, prostate and colon cancer in both humans and animals (Shahidi and Ambigaipalan, 2018). The data obtained in this work showed that the Hi prepupae meal dietary inclusion determined an increase in this ratio, which varied in larvae from ~0.46 in group Hi0 to ~1.0 in group Hi100, in juveniles from ~0.41 in group Hi0 to ~1.5 in group Hi100, in adult males from ~0.43 in the Hi0 group to ~1.6 in the Hi100 group, and in adult females from ~0.37 in the Hi0 group to ~1.6 in the Hi100 group. Thus, although the n6/n3 ratio values increased, they remained well below the maximum value of 5 expected for a healthy diet.

5. Conclusions

The approach suggested in this work, namely the study of the absolute content of FA in zebrafish, instead of the generally used FA percentage vs total FAs, has been found to be very useful to study the real variations of the FA composition in fish in relation to the composition of the feed and to the life stage. This study demonstrated a different content and profile of FAs between the investigated life stages of zebrafish, showing that the composition of FAs in the experimental model *Danio rerio* was influenced by the composition of the diet, as well as by the life stage of the fish. The study focused only on FAs profile of zebrafish fed Hi-meal based diets and did not perform a multidisciplinary approach to evaluate the response of fish to such diets which was assessed in several previous studies. However, from the obtained results, it can be assumed that the use of PUFA-enriched Hi prepupae meal in substitution of FM and FO in appropriate percentages, can be considered in the aquaculture field for the purpose of a good compromise between environmental sustainability and fish welfare.

Notes

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CRediT authorship contribution statement

Cristina Truzzi: Conceptualization, Data curation, Funding acquisition, Investigation, Methodology, Resources, Supervision, Writing – original draft, Writing – review & editing. **Federico Girolametti:** Data curation, Formal analysis, Investigation, Methodology, Software, Visualization, Writing – original draft. **Anna Annibaldi:** Data curation. **Matteo Zarantoniello:** Methodology, Writing – review & editing. **Ike Olivotto:** Conceptualization, Funding acquisition, Project administration, Writing – review & editing. **Paola Riolo:** Methodology. **Francesca Tulli:** Methodology, Writing – review & editing. **Silvia Illuminati:** Supervision, Writing – review & editing.

Declaration of Competing Interest

Authors have no competing interests to declare.

Appendix A. Supporting information

Supplementary data associated with this article can be found in the online version at doi:10.1016/j.anifeedsci.2023.115761.

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