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Can Insect-Based Diets Affect Zebrafish (*Danio rerio*) Reproduction? A Multidisciplinary Study

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Abstract

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

Keywords: Black Soldier Fly, gene expression, fatty acid profile, *Hermetia illucens*, aquaculture

Introduction

Zebrafish (*Danio rerio*) is a well-established experimental model for studies in the reproduction field since it is easily reared in laboratory conditions and, if compared to other teleost species, it reaches sexual maturity in a relatively short time.[1–4] In addition, over the last years, it gained a primary importance in assessing the role of specific dietary nutrients, such as fatty acids, in the reproductive processes of teleost.[5,6]

Zebrafish is an asynchronous spawner and, in females, the complete maturation of a single oocyte takes place in ~10 days.[1,2,4] Studies on reproductive mechanisms in zebrafish are also eased by a well-consolidated knowledge on oocyte development that is conventionally subdivided into five main stages.[4,7] Specifically, premeiotic oocyte progenitor cells, called oogonia, represent a continuous supply of new oocytes by multiple meiotic divisions. Once the oogonia enter meiotic division, they become primary oocytes. In this phase, oocytes initially reside in nests (Stage IA) and subsequently move from the original location, and increase in size up to about 140 μm.

Afterward, somatic cells are recruited with the formation of a definitive follicle (Stage IB) in which the oocyte is surrounded by an outer theca layer and an inner granulosa one. In Stage II, oocytes greatly increase in size up to a diameter of about 340 μm and membrane-limited vesicles, called cortical alveoli, appear. In addition, in this stage, the oocyte lipid deposition initiate and induces the formation of oil droplets containing neutral lipids.[8] In Stage III (further divided in Stage IIIA and Stage IIIB), the uptake of a female-specific liver-derived glycolipoprotein, vitellogenin, supports the further follicle size increase up to a diameter of about 740 μm.[4,9]

This process, called vitellogenesis, plays an important role in oocyte growth and fixes the transition from previtellogenic

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oocytes (Stages I and II) to vitellogenic ones (from Stage III onward). At the end of this phase (Stage IIIB), oocytes acquire maturational competence, by responding to maturation hormone stimuli. Later, in Stage IV, yolk vesicles containing vitellogenin begin to fuse with each other and oil droplets became more evident. Later, the nuclear envelope undergoes a breaking process, the ooplasm becomes opaque upon visual examination, and the follicle reaches the diameter of more than 740–760 μm.[9]

Since maturation occurs in few hours, only a few follicles are usually found at this developmental stage in analyzed zebrafish ovaries. The final maturation phase, traditionally described as Stage V, implies the complete fusion of the yolk vesicles and the separation of the mature oocyte from the follicular cells. Oocytes in this phase (diameter up to 1 mm) are rarely observed in zebrafish ovary, since they are ovulated into the ovarian lumen and rapidly spawned. Following ovulation, a typical postovulatory follicle (POF), composed of follicular cells (granulosa and theca cells) without the oocyte, remains in the ovary.[10,11]

In natural and cultured fishes, a disruption in the reproductive process may often be observed.[12] In this case, females fail to complete maturation and ovulation due to the degeneration of vitellogenic oocytes before ovulation in a process known as follicular atresia.[13,14] A number of stressors, including food availability and quality, are known to potentially interfere with oogenesis and induce atresia of ovarian follicles,[15–19] and should be studied in a deeper way when testing new aquafeed ingredients.

The ongoing process of oocyte maturation is one of the most metabolic demanding activities in fish and the quality of maternal nutrition is of primary importance for the appropriate allocation of macromolecules into the oocytes and their maturation.[20–22] Nutritional imbalances are well known to be detrimental for oocyte development[23] and, for this reason, investigating whether a diet is able to correctly sustain fish reproduction or not is of primary importance.

Over the last decades, several dietary formulations have been adopted for zebrafish and some of them are now available in the market.[24–26] All these formulations mainly contain fish meal (FM) and fish oil (FO) because of their high nutritional value in terms of proteins, amino acid profile, and lipids. However, the European Community has recently addressed attention and investments in the search of alternative and more sustainable ingredients for aquafeed formulation. According to the EC Directive number 2008/98 on the optimization of waste management, the European Community encourages efforts in finding more responsible, sustainable, and innovative aquafeed ingredients based on the circular economy concept, without affecting fish needs, welfare, and reproduction.[25–27] On this regard, recent studies explored the use of insect meal for aquafeed formulation and several studies have already been performed on zebrafish.[28–30] Insects show several advantages, since most of them can be cultured on land produced by by-products,[31–34] and have a low environmental footprint in terms of land use, water consumption, and CO_2 production.[35–37]

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

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

Fatty acid composition of adult female carcasses and fertilized eggs was analyzed through gas chromatography, while real-time quantitative PCR (qRT-PCR) was used to evaluate the expression of genes involved in fish lipid metabolism in the liver. Finally, ovaries were submitted to histological analysis to assess oocyte maturation stages and the oil droplet percentage in class IV oocytes and to spectroscopic analysis (by Fourier Transform Infrared Microspectroscopy [FTIRM]) to analyze the macromolecular composition of class IV oocytes.

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 (Ancona, Italy; 84/94-A). Optimal rearing conditions (see further section for details) were applied throughout the study, and all efforts were made to minimize animal suffering by using an anesthetic (MS222; Sigma-Aldrich, Saint Louis, MO).

Fish diet production

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

Experimental diets were formulated to be isonitrogenous and isolipidic by replacing 25% (BSF25) and 50% (BSF50) of the FM/oil of the control diet, respectively.

All the diets were sieved to obtain a different granulometry as a function of fish size development (as reported in Feeding Schedule section). Diets (in triplicate subsamples) were analyzed for proximate composition and gross energy content

measured by an adiabatic bomb calorimeter (IKA C7000; Werke GmbH and Co., Staufen, Germany).[44] For details, see Table 1.

Fish

Zebrafish (*Danio rerio*) used in this experiment were obtained from a pathogen-free AB broodstock (self-produced and that undergoes regular veterinary checks during the year) as described in Santangeli *et al.*[45] Embryos were maintained for 48 h in a Tecniplast System (Varese, Italy), subjected to the following conditions: 28°C temperature, pH 7.0, NO_2 and NH_3 concentrations $<0.01\,mg\cdot L^{-1}$, NO_3 concentration $<10\,mg\cdot L^{-1}$, and photoperiod 14L/10D. After this period, embryos were gently collected, counted, and randomly divided into three experimental groups (in triplicate) according to the three test diets.

Experimental design

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

Feeding schedule

Starting from 5 dph to 12 months, fish were fed as follows: zebrafish fed FM/FO diet (Control); zebrafish fed the diet including 25% BSF full-fat prepupae meal (BSF25); and zebrafish fed the diet including 50% of BSF full-fat prepupae meal (BSF50). Feed particle sizes were <100 μm from 5 to 15 days post spawning (dps), 101–200 μm from 16 to 30 dps, 201–400 μm from 31 to 60 dps, and 401–600 μm from 61 dps until the end of the experiment.

Zebrafish were fed the experimental diets (2% body weight, BW) twice a day (total feed per day 4% BW) and, in addition, from 5 to 10 dph, all groups were fed (one feeding in the morning) on the rotifer *Brachionus plicatilis* ($5\ ind\cdot mL^{-1}$) according to Piccinetti *et al.*.[49]

Growth and GSI

For growth measurements, 10 female zebrafish per tank (30 per dietary group) were randomly collected at the end of the experiment (12 months) and individually measured and weighed. The standard length was determined by a sliding calliper (Measy 2000 Typ 5921; Swiss; precision: 0.1 mm) and wet weight by an OHAUS Explorer (OHAUS Europe GmbH, Greifensee, Switzerland) analytical balance (precision: 0.1 mg). Furthermore, ovaries from these fish (30 per dietary group) were individually weighted and the GSI was estimated, using the following formula: ([ovary weight/zebrafish BW] × 100).

Egg collection and hatching rate

For the egg collection, 12-month-old male and female zebrafish were randomly transferred to breeding tanks (Tecniplast System). Specifically, five females and seven males were daily transferred to the breeding tanks for spawning and

Table 1. Ingredient Composition, Proximate Analysis, and Gross Energy Content of the Test Diets

	Control	*BSF25*	*BSF50*
Ingredients (g/kg)			
FM, 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
Minerals and vitamins supplement[a]	20	20	20
Binder	20	20	20
L-methionine	0	1	1
Proximate composition (%)			
Moisture	4.2 ± 0.03	5.5 ± 0.18	5.3 ± 0.42
Crude protein (Nx6.25)	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
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

[a]Composition of mineral mix (g/kg diet): Ca HPO_4 *$2H_2O$, 27.5; K_2HPO_4, 19.0; NaCl, 6.1; MgO, 2.0; $FeCO_3$, 1.75; KI, 0.15; ZnO, 0.11; MnO, 0.07; $CuSO_4$, 0.02; sodium selenite, 0.002. Composition of vitamin mix (mg/kg diet): thiamine HCl, 40; riboflavin, 40; pyridoxine HCl, 40; cyanocobalamin, 0.2; niacin, 300; calcium pantothenate, 100; folic acid, 5; biotin, 3; choline chloride, 5000; myo-inositol, 1000; ascorbic acid, 2000; a-tocopheryl acetate, 250; menadione, 90; vitamin A retinyl palmitate, 40,000 IU/kg diet; vitamin D3 cholecalciferol, 2500 IU/kg diet.[29]

BSF, Black Soldier Fly; FM, fish meal; NFE, N-free extractive.

egg collection, over a 10-day period. From each breading tank (about 20–30 min after the onset of light), eggs were collected. Eggs were initially observed and counted under a stereomicroscope (Leica Wild M3B; Leica Microsystems, Nussloch, Germany) for the evaluation of total spawned eggs.

Only fertilized eggs that presented a well-developed blastodisc at 3 h after fertilization were properly stored for further analyses and used for the hatching rate calculation (hatching rate = hatched eggs/total fertilized eggs × 100%).

Fertilized eggs were transferred to 10 cm diameter Petri dishes containing E3 medium (5 mM NaCl, 0.17 mM KCl, 0.33 mM CaCl2, and 0.33 mM MgSO4, to pH 7.0) and incubated at 28°C.

More specifically, for the hatching rate evaluation, 150 embryos from each dietary treatment (in triplicate) were randomly selected and distributed among Petri dishes (50 embryos per dish) containing E3 medium and then maintained in the incubator at 28°C for 5 days postfertilization. Hatched larvae were finally counted.

Lipid extraction and fatty acid analysis

Twelve-month-old female carcasses (3 fish per tank and 9 per dietary group) and just fertilized eggs (3 pools of 300 eggs per tank and 9 pools per dietary group) were analyzed for lipid content and fatty acid composition. Samples were minced, homogenized (homogenizer MZ 4110; DCG Eltronic, Monza, Italy), freeze-dried (Edwards EF4, Crawley, Sussex, England), and lipid extraction was carried out on lyophilized powders following a microwave-assisted extraction.[50,51] Fatty acid methyl esters (FAMEs) were prepared according to Truzzi and colleagues,[52] 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. A CPS ANALITICA CC-wax-MS (30 m × 0.25 mm inner diameter and 0.25 μm film thickness) capillary column was used to separate FAMEs. Instrumental conditions for the studied matrices were set up, according to Truzzi *et al.*[53,54] For each analyzed aliquot of sample, at least three runs were performed on the gas chromatography-mass spectrometry (GCMS).

Histological analysis

Ovaries collected from 5 different zebrafish specimens per tank (15 per dietary group) were used for the histological analysis and analyzed according to Migliaccio *et al.*[55] Briefly, after fixation in Bouin' solution (Sigma-Aldrich, Milano, Italy; 4C° for 24 h), samples were washed three times, for 10 min each, with ethanol (70%) and stored in the same ethanol solution. Samples were then dehydrated in increasing ethanol solutions (80%, 95% and 100%), washed with xylene (Bio-Optica, Milano, Italy), and embedded in paraffin (Bio-Optica). Solidified paraffin blocks were cut with a microtome (Leica RM2125 RTS; Leica) and each ovary was fully sectioned.

Five micrometer sections were stained with Mayer hematoxylin and eosin Y (Sigma-Aldrich, Milano, Italy) and observed using a Zeiss Axio Imager.A2 (Oberkochen, Germany) microscope to count the number of previtellogenic, class III, class IV, POF, and atretic oocytes based on their morphological features.[55] Images were acquired by means of a combined color digital camera Axiocam 503 (Zeiss) and all the sections were analyzed. Oocyte count was performed by using ZEN 2.3 lite Software (Zeiss). To minimize repetition in oocyte count, oocytes were marked in each section. Results were reported as the percentage of previtellogenic, classes III and IV oocytes on the total of oocytes counted. To further evaluate the histological features of class IV oocytes, the percentage of ooplasm area occupied by oil droplets (oil droplets area = ODA) at this stage was analyzed. For the analysis of ODA, class IV oocytes of each dietary group ($n = 9$/group) from three different ovaries per group were analyzed. Specifically, histological pictures of class IV oocytes with a diameter ranging from 700 to 740 μm were acquired by mean of ZEN 2.3 lite Software (Zeiss) and the area outside the oocyte was not acquired to avoid measurements on false positive areas (e.g., see Fig. 8b). Images were then analyzed by means of the software ImageJ. A homogeneous threshold was used to evaluate the white area in ooplasm (ODA) and data were reported as the mean (± standard deviation [SD]) of the observations.

Fourier Transform Infrared Microspectroscopy

FTIRM measurements were carried out by means of a Bruker Invenio interferometer, coupled with a Hyperion 3000 Vis-IR microscope and equipped with a MCT (Hg-Cd-Te) detector operating at liquid nitrogen temperature (Bruker Optics, Ettlingen, Germany).

Ovaries were collected from zebrafish specimens (two per tank, and hence six per dietary group) and stored at −80°C until they were cut by means of a cryomicrotome. For each sample, three sections (10 μm thick) were cut at 2 mm distance from each other. Sections were immediately deposited without any fixation process onto CaF_2 optical windows (1 mm thick and 13 mm in diameter), and then air-dried for 30 min.[56] Sections were observed by means of a 15× condenser/objective, to select class IV oocytes to be analyzed by FTIRM. On the inner compartment (excluding oolemma, nucleus, and zona Radiata) of each selected oocyte, ∼20 infrared (IR) spectra were acquired in transmission mode in the mid-infrared (MIR) range (4000–800 cm^{-1}; spectral resolution 4 cm^{-1}; spatial resolution 50 × 50 μm; 128 scans).[57,58] Before each acquisition, the background spectrum was acquired on a clean portion of the CaF_2 optical window. Raw IR spectra were preprocessed by Atmospheric Compensation (to correct for the atmospheric contributions of carbon dioxide and water vapor) and Vector Normalization (applied on the full frequency range, to avoid any artifact due to section thickness variations) routines (OPUS 7.5 software package; Bruker Optics).

IR spectra were then converted in second derivative mode (Savitzky-Golay filter, 9-point smoothing) and submitted to Principal Component Analysis (PCA) both alone and coupled with Linear Discriminant Analysis (PCA-LDA; OriginPro 2018b software; OriginLab Corporation, Northampton, MA). PCA was first used to reduce redundant information from the spectral dataset, describing each spectrum with a subset of selected principal components (PCs), explaining 95% of cumulative variance; then, the reduced spectra were used as input variables for LDA.[59]

On preprocessed IR spectra, meaningful band area ratios were calculated, as ratio between the integrated areas of

Table 2. Oligonucleotide Primers, and *ZFIN ID* (*ZDB-GENE*) of Each Gene Investigated in This Study

Gene	*Forward primer (5′-3′)*	*Reverse primer (5′-3′)*	*ZDB-GENE*
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
arpc1a (*hk*)	5′-CTGAACATCTCGCCCTTCTC-3′	5′-TAGCCGATCTGCAGACACAC-3′	ZDB-GENE-040116-1
rpl13 (*hk*)	5′-TCTGGAGGACTGTAAGAGG TATGC-3′	5′-AGACGCACAATCTTGAGAG CAG-3′	ZDB-GENE-031007-1

arpc1a and *rpl13* were used as housekeeping genes (*hk*).
Arpc1a, actin-related protein 2/3 complex, subunit 1A; *rpl13*, ribosomal protein L13.

specific IR bands. Instead, the integrated areas of the following spectral regions were calculated (Integration routine, Mode B, OPUS 7.5 software package; Bruker Optics): 3050–3000 cm^{-1} (representative of =CH groups in unsaturated lipid alkyl chains, named CH); 3000–2824 cm^{-1} (representative of CH_2 and CH_3 groups in lipid alkyl chains, named LIP); 1767–1710 cm^{-1} (representative of C=O groups in fatty acids, named FA); 1710–1478 cm^{-1} (representative of Amide I and II bands of proteins, named PRT); 1478–1426 cm^{-1} (representative of CH_2 groups in saturated lipid alkyl chains, named CH2); 1426–1358 cm^{-1} (representative of COO^- groups in amino acids, named COO); 1290–1188 cm^{-1} (representative of phosphate groups, named PH); 1188–1141 cm^{-1} (representative of COH groups in glycosylated compounds, named COH), and 1141–992 cm^{-1} (representative of phosphates and carbohydrates, named PH-CARBO).

RNA extraction and complementary DNA synthesis

Liver samples from 5 different female zebrafish from each tank (15 fish per dietary group) were sampled and then stored at −80°C for molecular analysis. Analyses were performed according to Olivotto *et al.*[60] Briefly, total RNA extraction from each sample was optimized using RNAzol® RT reagent (R4533; Sigma-Aldrich) following the manufacturer's instructions. Total RNA was eluted in 20 μL of RNase-free water (Qiagen, Venlo, Holland). Final RNA concentration was determined by the NanoPhotometer® P-Class (Implen, München, Germany). RNA integrity was verified by GelRed™ staining of 28S and 18S ribosomal RNA bands on 1% agarose gel. RNA was stored at −80°C until use. Finally, 2 μg of RNA was used for complementary DNA (cDNA) synthesis, employing the High Capacity cDNA Reverse Transcription Kit (Bio-Rad, Milan, Italy) following the manufacturer's instructions.

Real-time polymerase chain reaction

Polymerase chain reactions (PCRs) were performed with SYBER Green in an iQ5 iCycler thermal cycler (both from Bio-Rad) following Zarantoniello *et al.*[39]

Relative quantification of the expression of genes involved in long-chain PUFA biosynthesis (*elovl2*, *elovl5* and *fads2*) was performed. Actin-related protein 2/3 complex, subunit 1A (*arpc1a*), and ribosomal protein L13 (*rpl13*) were used as internal standards in each sample to standardize the results by eliminating variation in messenger RNA (mRNA) and cDNA quantity and quality. No amplification products were observed in negative controls and no primer-dimer formations were observed in the control templates. Amplification products were sequenced, and homology was verified. The data obtained were analyzed using the iQ5 optical system software version 2.0 (Bio-Rad), including GeneEx Macro iQ5 Conversion and genex Macro iQ5 files. Primer sequences were designed using Primer3 (210 v. 0.4.0) starting from zebrafish sequences available in ZFIN (https://zfin.org). Primer sequences used were reported in Table 2.

Statistical analysis

Data obtained were analyzed by one-way analysis of variance (ANOVA), with diet as the explanatory variable. All ANOVA tests were followed by Tukey's *post hoc* test. The statistical software package Prism6 (GraphPad Software, Inc.) was used. Significance was set at $p<0.05$ and all the results are presented as mean ± SD.

Results

Biometry

The standard length of adult zebrafish females was 27.7 ± 4.0 mm for BSF25 and 26.7 ± 2.3 mm for BSF50 (respectively) and resulted to be significantly lower compared to the Control (32.2 ± 3.0 mm; $p<0.05$) (Fig. 1a). Conversely, as concerned with both wet weight (0.7 ± 0.1, 0.7 ± 0.1, and 0.6 ± 0.1 g for Control, BSF25, and BSF50, respectively) (Fig. 1b) and GSI (10.8% ± 0.8%, 10.7% ± 1.5%, and 10.0% ± 2.3% for Control, BSF25, and BSF50, respectively), no differences ($p>0.05$) were detected among the experimental groups (Fig. 1c).

Spawned eggs and hatching rate

As reported in Table 3, no significant differences were observed between both daily and total number of spawned eggs. In particular, the number of daily spawned eggs was 192 ± 37 for Control and 190 ± 48 for BSF25, and the number of total spawned eggs was 1892 ± 53 for Control and 1903 ± 94 for BSF25. Conversely, a significant reduction of both daily and total spawned eggs was observed in BSF50 group (daily spawned eggs 81 ± 42 and total spawned eggs 811 ± 36) compared to the other experimental groups.

As regard to the hatching rate (Fig. 2), no differences ($p>0.05$) were detected among the experimental groups (percentage of hatching eggs of 74.4 ± 2.6 for Control, 75.3 ± 2.6 for BSF25, and 73.2 ± 2.2 for BSF50).

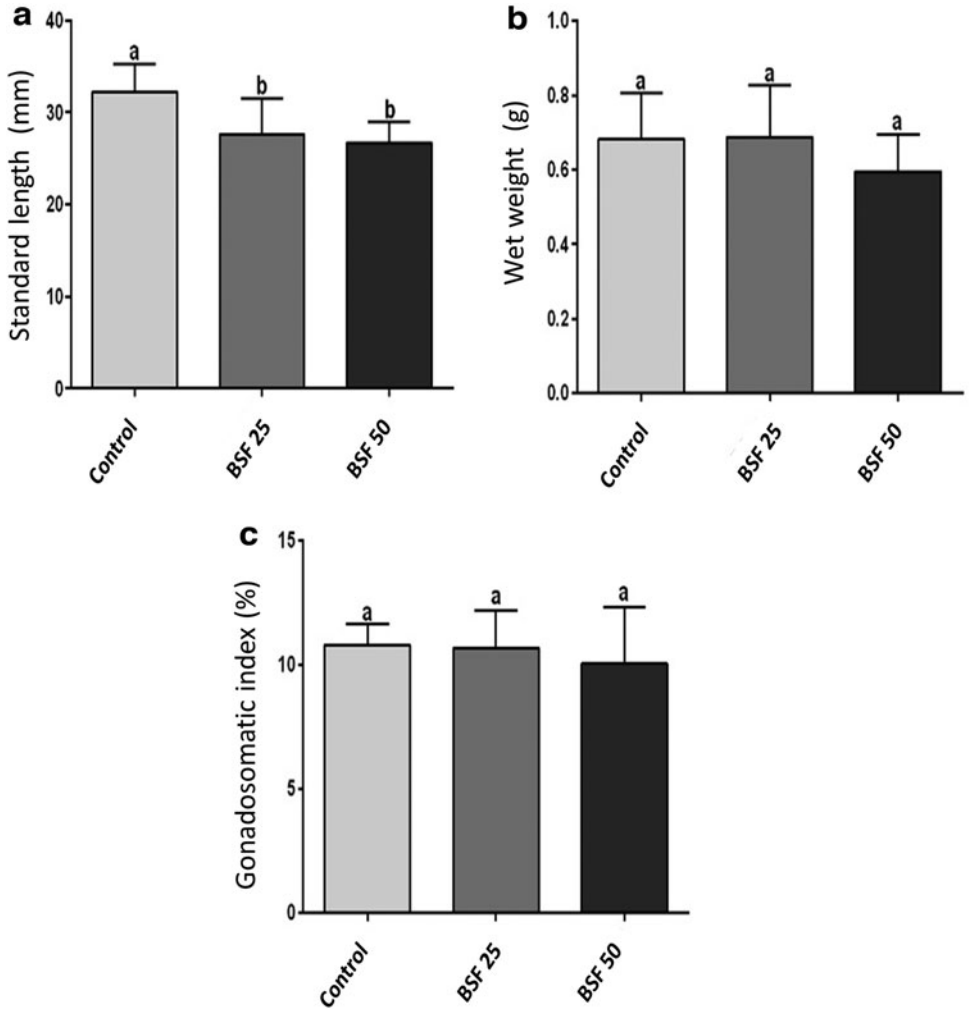


FIG. 1. Biometric measurements of 12-month-old female zebrafish. **(a)** Standard length; **(b)** wet weight; **(c)** gonadosomatic index. Zebrafish fed diet based on FM (Control) and diets with 25% (BSF25) or 50% (BSF50) replacement of FM with BSF meal. *Different letters* indicate statistically significant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

Fatty acid content and composition

Adult female carcasses. The FA content (as % of total FAs) of 12-month-old female zebrafish fed the different diets was affected by increasing inclusion levels of BSF full-fat prepupae meal in the diets (Fig. 3a). Specifically, a significant increase ($p < 0.05$) of SFA content was detected in BSF25 (27.5% ± 0.4%) and BSF50 (33.7% ± 0.4%) groups with respect to Control (25.8 ± 0.4).

Regarding mono-unsaturated-fatty acids (MUFA) and n6, both BSF25 (MUFA = 40.3% ± 0.7% and n6 = 15.9% ± 0.1%) and BSF50 (MUFA = 39.0% ± 0.4% and n6 = 16.3% ± 0.2%) groups showed a significantly ($p < 0.05$) higher percentage with respect to Control (MUFA = 35.2% ± 0.4% and

Table 3. Mean ± Standard Deviation of the Number of Total and Daily Spawned Eggs per Breeding Tank in a 10-Day Period

No. of eggs	*Control*	*BSF25*	*BSF50*
Per day	192 ± 37	190 ± 48	81 ± 42[a]
Total	1892 ± 53	1903 ± 94	811 ± 36[a]

Mean hatching rate per experimental group. Zebrafish fed diet based on FM (Control) and diets with 25% (BSF25) or 50% (BSF50) replacement of FM with BSF meal.

[a]Indicate significant difference among the groups ($p < 0.05$).

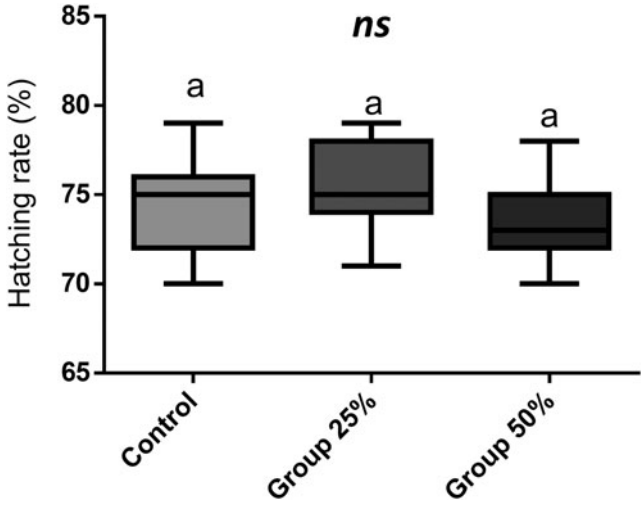


FIG. 2. Mean hatching rate per experimental group. Zebrafish fed diet based on FM (Control) and diets with 25% (BSF25) or 50% (BSF50) replacement of FM with BSF meal. *ns*, Differences among experimental groups are not significant ($p > 0.05$) (a).

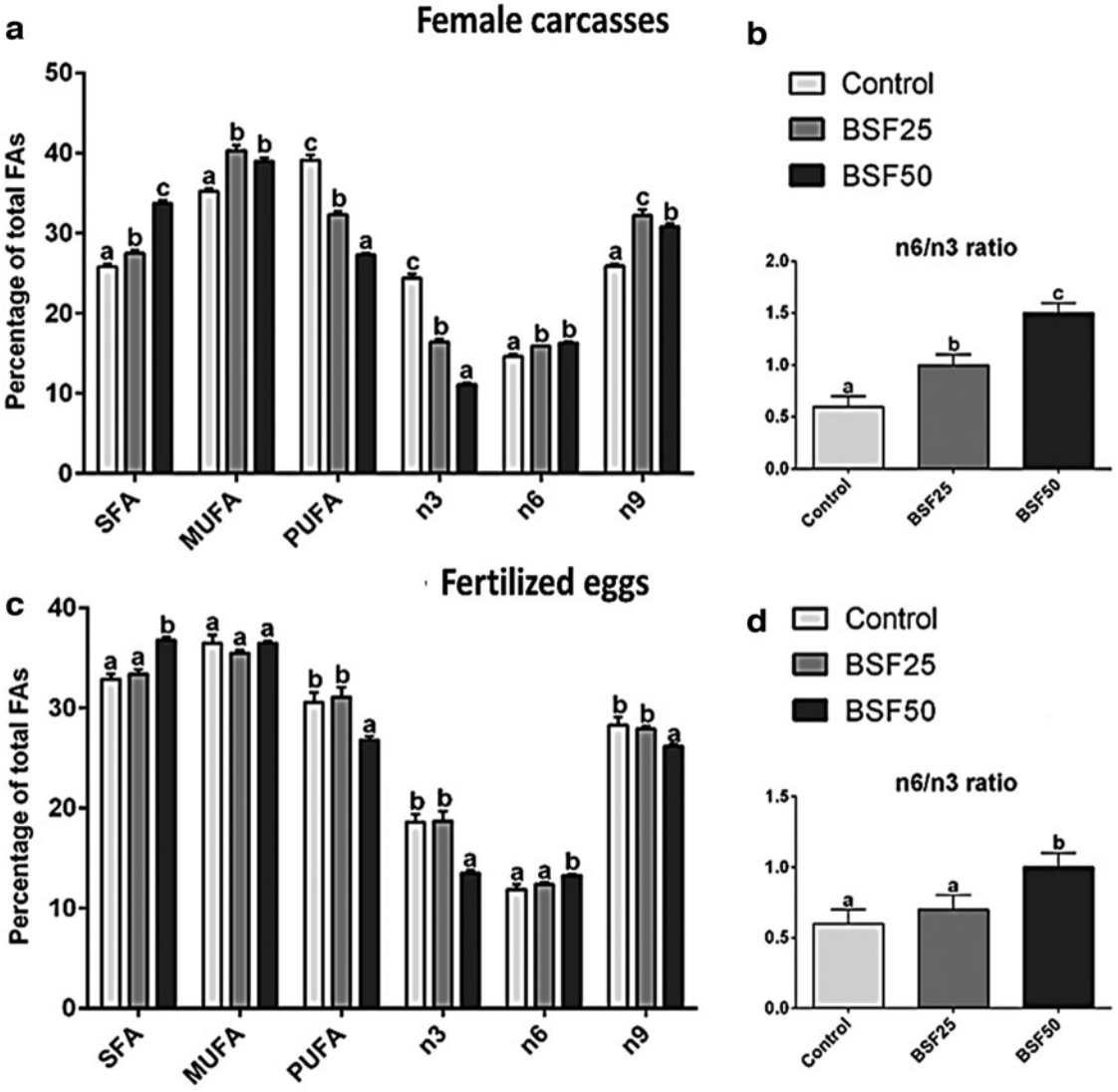


FIG. 3. Content of SFA, MUFA, and PUFA (as % of total FA) and contribution of omega 3 (n3), omega 6 (n6), and omega 9 (n9) fatty acids to lipid profile. **(a, b)** Twelve-month-old zebrafish females carcasses (Control, BSF25, and BSF50); **(c, d)** just fertilized eggs obtained from Control, BSF25, and BSF50 fish groups. *Different letters* indicate statistically significant differences among experimental groups compared within the same fatty acid class ($p < 0.05$). Values are presented as mean ± SD ($n = 12$). PUFA, polyunsaturated fatty acid; SFA, saturated fatty acid.

n6 = 14.6% ± 0.3%), while no differences ($p > 0.05$) between them were detected. The increasing inclusion levels of BSF full-fat prepupae meal in the diets resulted in a significant decrease ($p < 0.05$) of both PUFA (39.1% ± 0.6% for the Control, 32.3% ± 0.4% for the BSF25, and 27.3% ± 0.3% for BSF50; $p < 0.05$) and n3 content (24.4% ± 0.5% for the Control, 16.4% ± 0.4% for the BSF25, and 11.1% ± 0.2% for the BSF50 $p < 0.05$). Differently, considering n9 content, BSF25 (32.2% ± 0.7%) and BSF50 groups (30.8% ± 0.4%) evidenced higher ($p < 0.05$) percentages with respect to Control (25.9% ± 0.3%). Finally, as presented in Figure 3b, the higher the BSF full-fat prepupae inclusion in the diets, the higher was the n6/n3 ratio (0.6 ± 0.1 for Control, 1.0 ± 0.1 for BSF25, and 1.5 ± 0.1 for BSF50; $p < 0.05$).

Considering SFA composition in the three experimental groups (Table 4), the predominant SFA 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 experimental groups. BSF50 group showed a significantly ($p < 0.05$) higher content of Lauric (12:0) with respect to BSF25 and Control groups. The most represented MUFA was oleic acid (18:1n9) and its content was significantly ($p < 0.05$) higher in BSF25 and BSF50 groups with respect to Control. Finally, linoleic acid (18:2n6) was the predominant PUFA, with significantly ($p < 0.05$) higher percentages in BSF25 and BSF50 fish with respect to Control. Groups fed on BSF-based diets showed a significantly ($p < 0.05$) lower eicosapentaenoic acid (EPA; 3.0% ± 0.3% for BSF25 and 2.3% ± 0.4% for BSF50) and docosahexaenoic acid (DHA; 8.3% ± 0.9% for BSF25 and 4.6% ± 1.1% for BSF50) content than Control (6.0% ± 0.8% for EPA and 14.3% ± 0.9% for DHA).

Fertilized eggs. The FA content (as % of total FAs) of just fertilized eggs from the three experimental groups is

Table 4. Fatty Acid Composition (% Fatty Acid Methyl Esters) of Adult Female Carcasses and Just Fertilized Eggs

	Females carcasses			*Fertilized eggs*		
	Control	*BSF25*	*BSF50*	*Control*	*BSF25*	*BSF50*
10:0	n.d.	0.03 ± 0.01^{a}	0.08 ± 0.01^{b}	n.d.	0.04 ± 0.01^{a}	0.1 ± 0.01^{b}
12:0	0.2 ± 0.1^{a}	4.4 ± 0.4^{b}	8.9 ± 1.1^{c}	0.3 ± 0.1^{a}	2.0 ± 0.2^{c}	1.6 ± 0.1^{b}
13:0	0.03 ± 0.01^{a}	0.02 ± 0.01^{a}	0.03 ± 0.01^{a}	0.01 ± 0.01^{a}	0.01 ± 0.01^{a}	0.02 ± 0.01^{a}
14:0	2.7 ± 0.1^{a}	2.8 ± 0.2^{a}	4.2 ± 0.6^{b}	1.7 ± 0.1^{a}	2.3 ± 0.1^{b}	3.1 ± 0.2^{c}
14:1n5	0.1 ± 0.1^{a}	0.3 ± 0.1^{ab}	0.5 ± 0.1^{b}	0.1 ± 0.1^{a}	0.1 ± 0.1^{a}	0.2 ± 0.1^{a}
15:0	0.5 ± 0.1^{a}	0.4 ± 0.1^{a}	0.4 ± 0.1^{a}	0.5 ± 0.1^{a}	0.4 ± 0.1^{a}	0.5 ± 0.1^{a}
15:1n5	0.2 ± 0.1^{a}	0.3 ± 0.1^{a}	0.3 ± 0.1^{a}	0.4 ± 0.1^{a}	0.3 ± 0.1^{a}	0.4 ± 0.1^{a}
16:0	16.9 ± 0.1^{a}	16.2 ± 1.6^{a}	17.1 ± 1.0^{a}	23.6 ± 0.9^{ab}	22.0 ± 0.1^{a}	24.3 ± 1.0^{b}
16:1n9	1.0 ± 0.1^{a}	1.3 ± 0.3^{a}	1.3 ± 0.4^{a}	2.4 ± 0.1^{ab}	2.6 ± 0.1^{b}	2.2 ± 0.1^{a}
16:1n7	4.7 ± 0.6^{a}	4.0 ± 0.3^{a}	4.2 ± 0.1^{a}	3.7 ± 0.1^{a}	3.8 ± 0.1^{a}	4.9 ± 0.1^{b}
17:0	0.7 ± 0.1^{b}	0.5 ± 0.1^{ab}	0.4 ± 0.1^{a}	0.6 ± 0.1^{a}	0.5 ± 0.1^{a}	0.7 ± 0.1^{a}
17:1n7	0.7 ± 0.1^{a}	0.6 ± 0.1^{a}	0.5 ± 0.1^{a}	0.6 ± 0.1^{a}	0.6 ± 0.1^{a}	0.8 ± 0.1^{a}
18:0	4.4 ± 0.3^{c}	2.9 ± 0.1^{b}	2.4 ± 0.1^{a}	6.1 ± 0.2^{a}	6.1 ± 0.1^{a}	6.3 ± 0.2^{a}
18:1n9	23.4 ± 1.9^{a}	29.9 ± 2.2^{b}	28.8 ± 1.4^{b}	24.2 ± 0.2^{b}	24.4 ± 0.2^{b}	23.1 ± 0.4^{a}
18:1n7	3.5 ± 0.2^{b}	2.9 ± 0.2^{a}	2.6 ± 0.1^{a}	3.5 ± 0.1^{b}	2.8 ± 0.1^{a}	4.0 ± 0.1^{c}
18:2n6	11.7 ± 0.1^{a}	13.2 ± 0.8^{b}	13.2 ± 0.2^{b}	8.5 ± 0.1^{a}	8.8 ± 0.1^{b}	8.9 ± 0.1^{b}
18:3n6	0.3 ± 0.1^{a}	0.1 ± 0.1^{a}	0.1 ± 0.1^{a}	0.2 ± 0.1^{a}	0.4 ± 0.2^{a}	0.2 ± 0.1^{a}
18:3n3	3.6 ± 0.3^{a}	4.7 ± 1.3^{a}	3.9 ± 0.8^{a}	3.0 ± 0.1^{b}	3.0 ± 0.2^{b}	1.2 ± 0.1^{a}
20:0	0.2 ± 0.1^{a}	0.1 ± 0.1^{a}	0.1 ± 0.1^{a}	0.2 ± 0.1^{a}	0.1 ± 0.1^{a}	0.1 ± 0.1^{a}
20:1n9	0.3 ± 0.1^{a}	0.4 ± 0.2^{a}	0.3 ± 0.1^{a}	1.6 ± 0.1^{b}	0.8 ± 0.1^{a}	0.9 ± 0.1^{a}
20:2n6	0.6 ± 0.1^{a}	0.6 ± 0.1^{a}	0.7 ± 0.1^{a}	1.1 ± 0.1^{b}	0.7 ± 0.1^{a}	0.9 ± 0.1^{ab}
20:3n6	0.6 ± 0.0^{a}	0.7 ± 0.2^{ab}	1.0 ± 0.1^{b}	1.5 ± 0.1^{a}	1.5 ± 0.1^{a}	1.8 ± 0.1^{b}
21:0	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.
20:4n6	1.4 ± 0.2^{a}	1.3 ± 0.3^{a}	1.4 ± 0.2^{a}	0.8 ± 0.1^{a}	1.0 ± 0.1^{a}	1.4 ± 0.2^{b}
20:3n3	0.5 ± 0.2^{a}	0.4 ± 0.1^{a}	0.3 ± 0.1^{a}	1.0 ± 0.1^{c}	0.6 ± 0.1^{b}	0.3 ± 0.1^{a}
20:5n3	6.0 ± 0.8^{b}	3.0 ± 0.3^{a}	2.3 ± 0.4^{a}	4.2 ± 0.2^{b}	4.5 ± 0.1^{b}	2.8 ± 0.1^{a}
22:0	0.15 ± 0.02^{b}	0.09 ± 0.02^{a}	0.07 ± 0.02^{a}	0.05 ± 0.01^{b}	0.03 ± 0.01^{ab}	0.02 ± 0.01^{a}
22:1n9	1.0 ± 0.2^{b}	0.5 ± 0.1^{a}	0.4 ± 0.1^{a}	0.16 ± 0.01^{c}	0.08 ± 0.03^{b}	0.03 ± 0.01^{a}
24:0	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.
22:6n3	14.3 ± 0.9^{b}	8.3 ± 0.9^{b}	4.6 ± 1.1^{a}	10.4 ± 0.4^{b}	10.6 ± 0.5^{b}	9.2 ± 0.1^{a}
24:1n9	0.2 ± 0.1^{a}	0.1 ± 0.1^{a}	0.0 ± 0.1^{a}	n.d.	n.d.	n.d.

Fatty acid composition (% fatty acid methyl esters) of adult female carcasses and just fertilized eggs from the different experimental groups. For each matrix, mean within rows bearing *different letters* is significantly different ($p < 0.05$). Female carcasses: fish fed diet based on FM (Control) and diets with 25% (BSF25) or 50% (BSF50) replacement of FM with BSF meal. For FA composition of experimental diets, see Zarantoniello *et al.*[29]

presented in Figure 3c. Considering SFA and n6, BSF50 group showed a significantly ($p < 0.05$) higher percentage (SFA = 36.8% ± 0.3% and n6 = 13.3% ± 0.1%) with respect to Control (SFA = 32.9% ± 0.5% and n6 = 11.9% ± 0.5%) and BSF25 group (SFA = 33.4% ± 0.5% and n6 = 12.4% ± 0.2%). As concerns MUFA, no significant differences ($p > 0.05$) were detected among the experimental groups (36.5% ± 0.8% for the Control, 35.5% ± 0.3% for BSF25, and 36.5% ± 0.2% for BSF50). Regarding PUFA, n3, and n9, BSF50 group (26.8% ± 0.4%, 13.5% ± 0.3%, and 26.2% ± 0.2%, respectively) showed significantly ($p < 0.05$) lower percentages with respect to Control (30.6% ± 1.0%, 18.6% ± 0.8%, and 28.3% ± 0.8%, respectively) and BSF25 group (31.1% ± 1.0%, 18.7% ± 1.0%, and 27.9% ± 0.3%, respectively). Finally, as concerns the n6/n3 ratio (Fig. 3d), Control (0.6% ± 0.1%) and BSF25 (0.7% ± 0.1%) groups were characterized by a significantly ($p < 0.05$) lower value with respect to BSF50 group (1.0% ± 0.1%).

With regard to specific FA composition (% FAMEs) (Table 4), the most relevant SFA in all the experimental diets was the palmitic acid (16:0). Lauric acid (C12) content was significantly ($p < 0.05$) higher in fertilized eggs spawned by fish fed on BSF-based diets with respect to control. Oleic acid (18:1n9) was the most abundant MUFA in eggs from all the dietary treatments. Finally, just fertilized eggs from BSF50 group showed a significantly ($p < 0.05$) lower EPA and DHA content (2.8% ± 0.1% and 9.2% ± 0.1%) with respect to Control (4.2% ± 0.2% and 10.4% ± 0.4%) and BSF25 (4.5% ± 0.1% and 10.6% ± 0.5%) groups, which did not present significant differences ($p > 0.05$) between them.

Histological analysis of ovaries

Histology performed on ovary sections of Control, BSF25, and BSF50 groups showed all the different classes of oocytes (Fig. 4). In particular, for the oocyte count, oocytes were assigned at three developmental stages: previtellogenic oocytes, with or without presence of cortical alveus (100–280 μm diameter) (Fig. 4a, b); class III oocytes, oocytes with enlarged yolk vesicles and vitellin membrane (280–740 μm diameter) (Fig. 4c); and class IV oocytes, oocytes without nuclear envelope and opaque ooplasm (>740 μm diameter) (Fig. 4d). Since class V oocytes are ovulated in few hours, they were rarely found in the analyzed zebrafish ovaries. After ovulation,

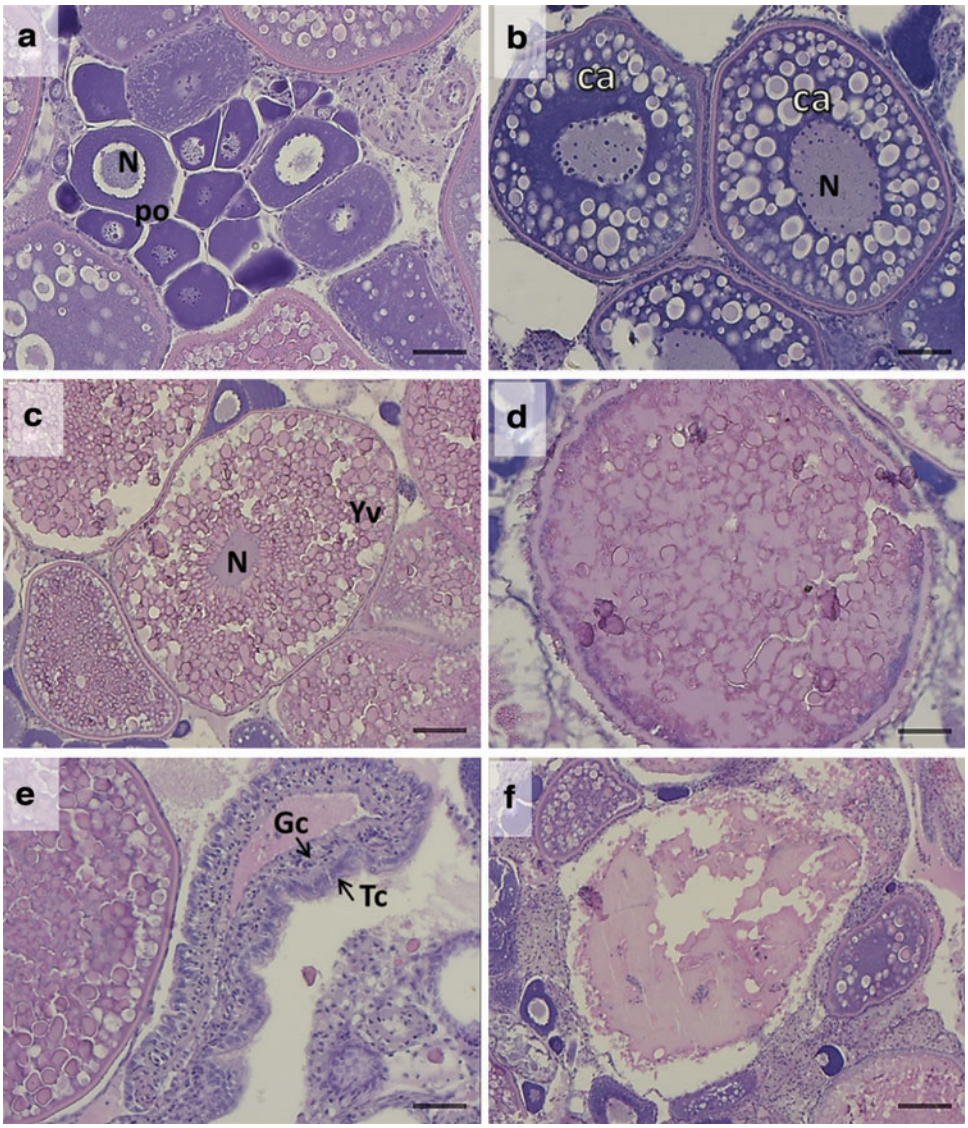


FIG. 4. Oocyte developmental stages in zebrafish ovary. Previtelloganic stages: **(a)** oogonia and primary oocytes lying in nest; **(b)** previtellogenic oocytes with cortical alveoli; **(c)** oocyte at stage III with yolk vesicles; **(d)** oocyte at stage IV; **(e)** oocyte at stage V; **(f)** POF. Ca, cortical alveoli; N, nucleus; po, primary oocytes; POF, postovulatory follicle; Yv, yolk vesicles. Scale: **(a, b)** 50 μm, **(c–e)** 100 μm, **(f)** 200 μm. Color images are available online.

follicular cells (theca and granulosa cells) remain in the ovary and constitute a characteristic histological structure called POF. For this reason, we decided to exclude class V oocytes from the analysis and addressed our analysis on the percentage of the POF number with respect to the total number of oocytes per ovary analyzed. Atretic oocytes were observed in particular in the BSF50 group. Atretic oocytes were identified as described in Üçüncü and Çakıcı[61] and were characterized by basal membrane disintegration, invagination and breakdowns of zona radiate, degeneration and absorption of ooplasm, and granulosa and theca cell hyperplasia (Fig. 5).

The POF number (expressed in percentage on the total counted oocytes) was also analyzed. POF was identified as collapsed empty follicles made of intact granulosa and theca cell layers, without oocyte. POF was observed in all the experimental groups and a high magnification representative image is reported in Figure 4f.

In Figure 6, representative histological sections of whole zebrafish ovaries are shown, while in Figure 7, the frequency of the different oocyte classes is presented. BSF50 group showed a significantly ($p<0.05$) higher percentage of previtellogenic oocytes (86.5% ± 4.5%) with respect to both Control (78.5% ± 3.5%) and BSF25 (76.5% ± 3.5%) groups, which did not evidence significant differences ($p>0.05$) between them (Fig. 7). Conversely, as concerns class III oocytes, Control and BSF25 groups showed a significantly ($p<0.05$) higher percentage (19.5% ± 3.5% and 20.5% ± 1.5%) than BSF50 group (4.5% ± 2.5%). With regard to class IV oocytes, no significant differences ($p>0.05$) were detected among the experimental groups (1.5% ± 0.5%, 1.5% ± 0.5%, and 1.5% ± 0.5% for Control, BSF25, and BSF50, respectively). On the contrary, a significant increase of atretic oocytes (%/total oocytes) was observed in BSF50 group (8.5 ± 1.5) compared to the Control (0.5% ± 0.5%) and BSF25 (1% ± 0.5%) groups. Representative images of the analysis of the area occupied by lipid droplets (ODA) on histological sections of class IV oocytes are shown in Figure 8a and b. Results evidenced a significant higher ODA in BSF50 group oocytes (20 ± 2.7% of the total ooplasm) compared to Control (6.3% ± 1.7%) and BSF25 groups (8.1 ± 9.1%) (Fig. 8c).

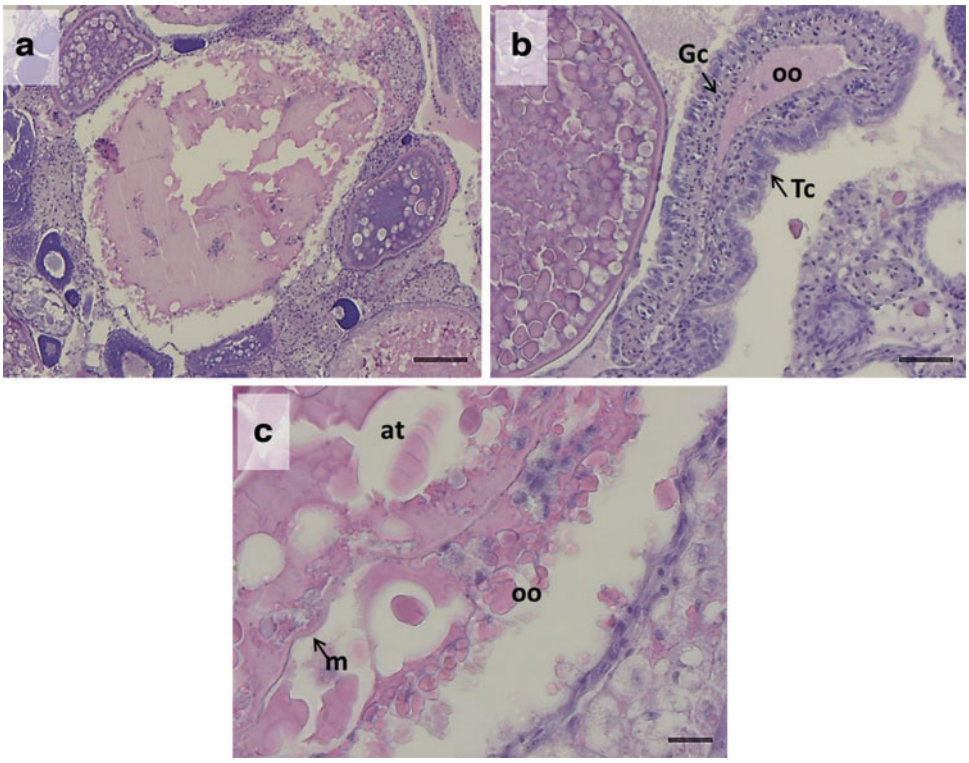


FIG. 5. Atretic oocyte. **(a)** Low magnification of a class V atretic oocyte; **(b)** iperplastic follicular theca (Tc) and granulosa (Gc) cells surrounding degenerating ooplasm (oo) of an atretic oocyte; **(c)** high magnification showing corrugated oocyte membrane (m), atretic zones (at), and degenerated expelled ooplasm. Scale: **(a)** 200 μm, **(b)** 100 μm, **(c)** 20 μm. Color images are available online.

FTIRM analysis

The inner compartment (excluding oolemma, nucleus, and zona radiata) of classes IV oocytes from 12-month-old Zebrafish females feeding diets based on FM (Control) and on 25% (BSF25) or 50% (BSF50) replacement of FM with BSF meal was investigated by FTIRM spectroscopy.

In Figure 9a, the average IR spectrum of a class IV oocyte is shown. Specific bands of the biochemical components of the sample were detected: 3015 cm^{-1} (stretching mode of = CH groups in lipid alkyl chains); 2959, 2927, and 2856 cm^{-1} (symmetric and asymmetric stretching modes of CH_2 and CH_3 groups in lipid alkyl chains); 1739 cm^{-1} (stretching mode of C = O groups in fatty acids); 1652 and 1541 cm^{-1} (Amide I and II bands of proteins); 1455 cm^{-1} (bending mode of CH_2 groups, mainly in lipids); 1396 cm^{-1} (stretching mode of COO^- groups in amino acids); 1237 cm^{-1} (asymmetric stretching mode of phosphate groups); 1156 cm^{-1} (stretching mode of COH groups in glycosylated compounds); and 1084 cm^{-1} (symmetric stretching mode of phosphate groups and stretching modes of C-O and C-C bonds in carbohydrates).

IR spectra of Control, BSF25, and BSF50 groups were submitted to PCA and PCA-LDA analyses to highlight the spectral features of this cell compartment in relationship with the different diets (Fig. 9b–e). With regard to PCA scores plot, no segregation pattern was found among all the experimental groups (Fig. 9b), even if the PC1 and PC2 loading spectra pinpointed changes in the regions related to lipids (zone 1), fatty acids (zone 2), and proteins (zone 3) (Fig. 9c). Conversely, PCA-LDA displayed a complete separation of all the three experimental groups (Fig. 9d), with LD1 and LD2 representing the first and second linear discriminant functions obtained by the canonical variable scores of PCA-

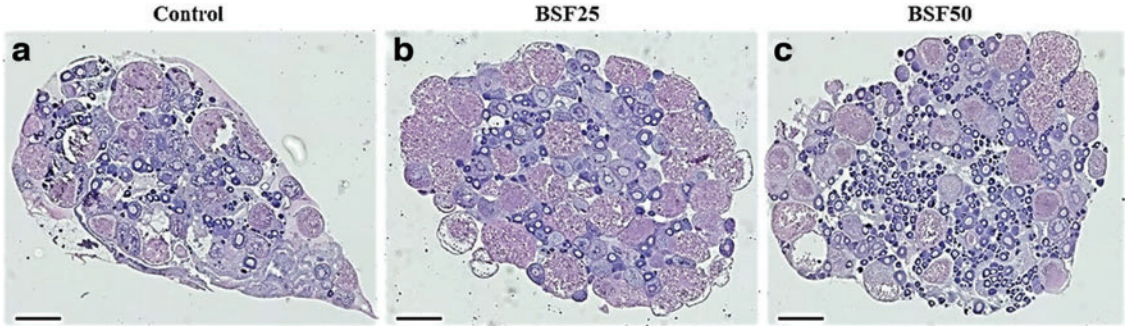


FIG. 6. Example of histomorphology of zebrafish ovaries. Zebrafish fed diet based on FM (Control) **(a)** and diets with 25% (BSF25) **(b)** or 50% (BSF50) **(c)** replacement of FM with BSF meal. Scale bars: 500 μm. Color images are available online.

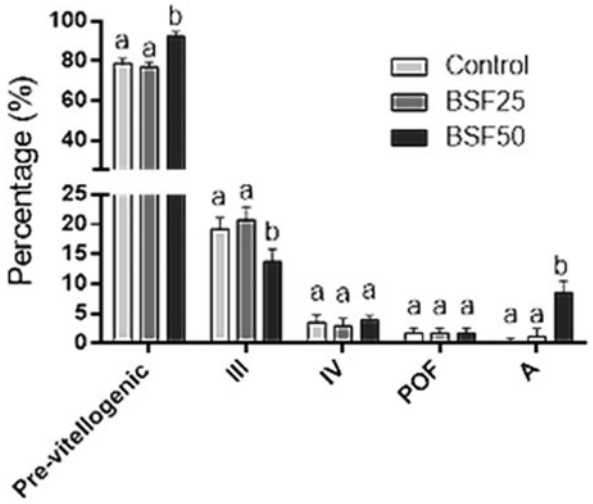


FIG. 7. Percentage of previtellogenic, class III, class IV, class V, atretic oocytes, and POF. Results are expressed in percentage ([number of oocytes in each stage/total oocytes] × 100) as mean ± SEM. *Different letters* indicate statistically significant differences among the groups ($p < 0.05$). SEM, standard error of the mean.

LDA. In particular, LD1 showed to discriminate between Control and the other two groups, while LD2 allowed to distinguish between BSF25 and BSF50 spectra. The one-dimensional score plot of LD1 and LD2 displayed in Figure 9e confirmed the importance of the two linear discriminant variables in distinguishing the three experimental groups.

To investigate the biochemical composition of the inner compartment of Control, BSF25, and BSF50 class IV oocytes, the following band area ratios were calculated and statistically analyzed (Fig. 10): LIP/CELL (relative amount of overall lipids); CH2/CELL (relative amount of SFAs); CH/CELL (relative amount of unsaturated fatty acids); FA/CELL(relative amount of overall fatty acids); PRT/CELL (relative amount of overall proteins); COO/CELL (relative amount of COO^- in amino acids); PH/CELL (relative amount of phosphates); COH/CELL (relative amount of glycosylated compounds); and PH-CARBO/CELL (relative amount of phosphates and carbohydrates). CELL, calculated as sum of the integrated areas of the regions 3000–2824 and 1780–950 cm^{-1}, was considered representative of the total cell biomass.

With regard to lipids, a statistically significant increase (LIP/CELL, $p < 0.05$) was found in BSF25 and BSF50 experimental groups; moreover, in BSF50, the increment in SFAs (FA/CELL, $p < 0.05$; CH2/CELL, $p < 0.05$) and the decrement

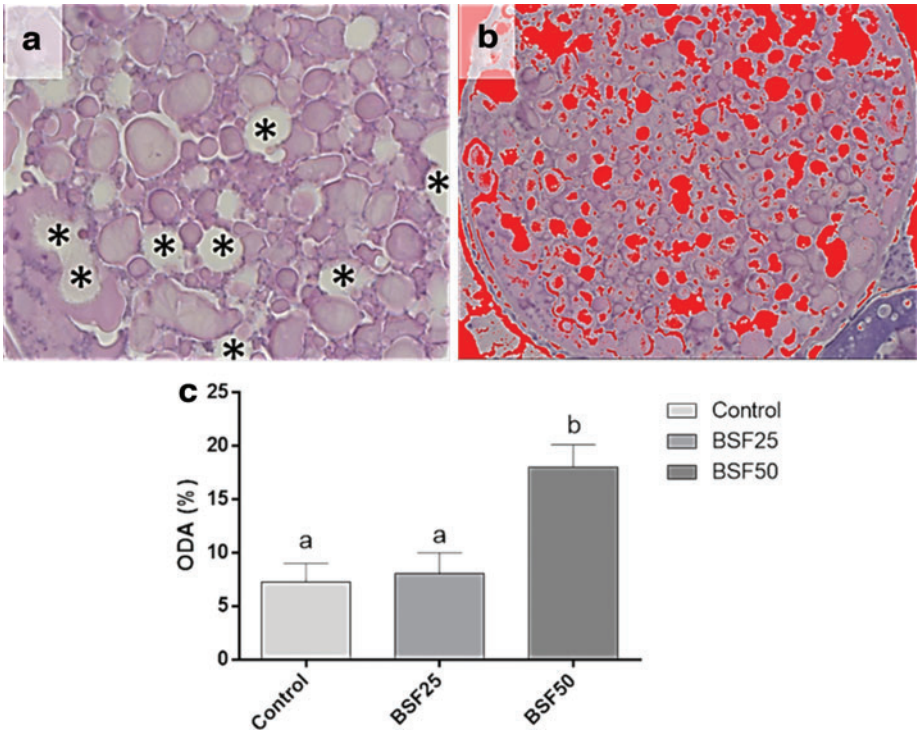
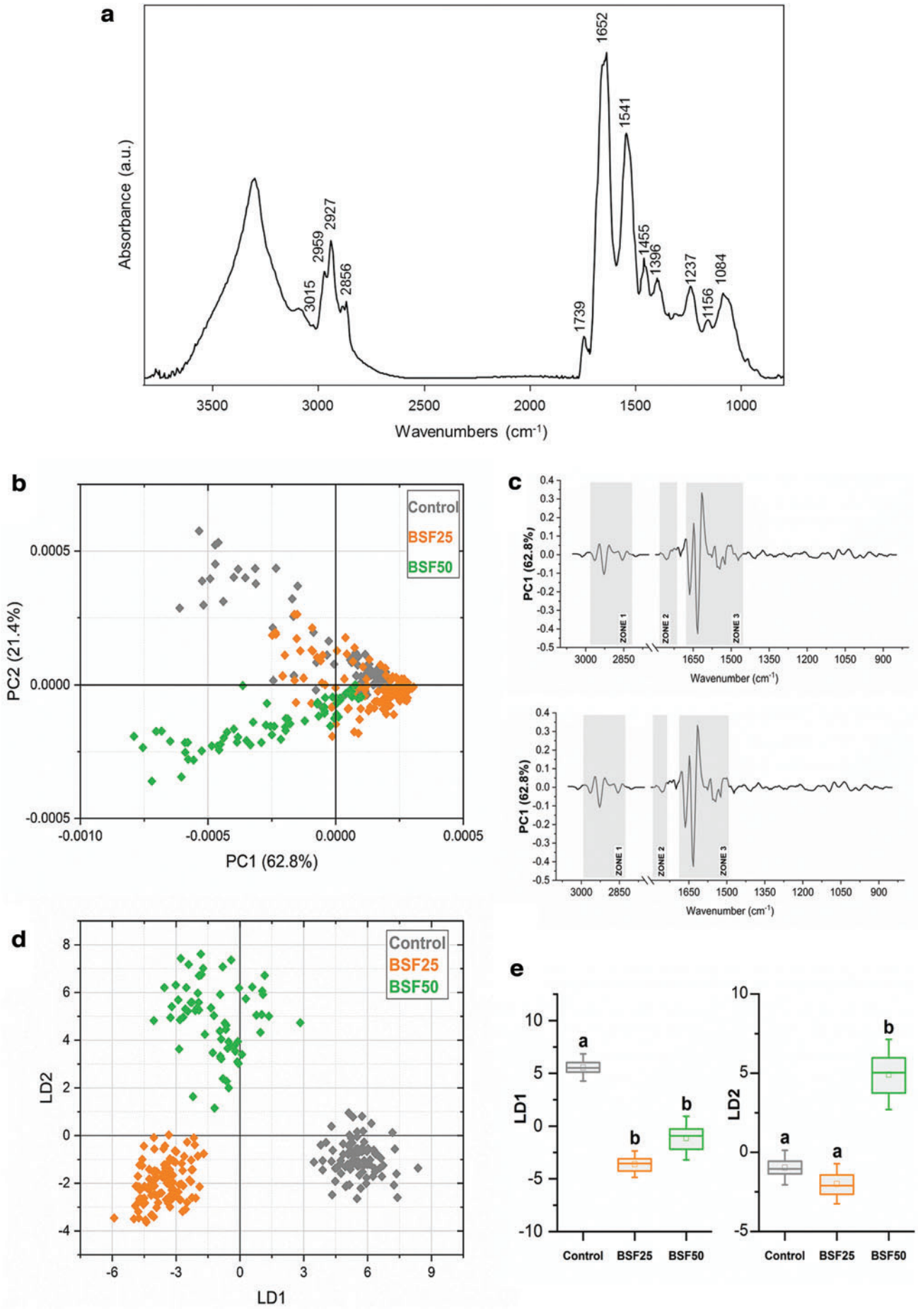


FIG. 8. Percentage of the area occupied by lipid droplets in class IV oocytes (ODA). **(a)** Histological representative section of a class IV oocyte with oil droplets (*asterisks* [*]); **(b)** example of analysis of the area occupied by oil droplets (ODA) performed with ImageJ software; **(c)** percentage of the area occupied by oil droplets. Results are representative of class IV oocyte sections ($n = 3$/ovary) from three different ovary (total 9/group) and expressed in percentage of ODA on the total ooplasm. *Different letters* indicate statistically significant differences among the groups ($p < 0.05$). ODA, oil droplet area. Color images are available online.

FIG. 9. **(a)** Average IR spectrum of class IV oocyte from Control group. The IR spectrum is shown in absorbance mode in the 3800–800 cm^{-1} range. The wavenumbers (cm^{-1}) of the most relevant IR bands are reported. **(b)** PCA scores plot of Control, BSF25, and BSF50 second-derivative spectra. The percentages of the variance explained by PC1 and PC2 are reported in *parentheses*. **(c)** Loading spectra of PC1 and PC2. **(d)** PCA-LDA score plot of Control, BSF25, and BSF50 second-derivative spectra. LD1 and LD2 represent the first and second linear discriminant functions obtained by the canonical variable scores of PCA-LDA. **(e)** One-dimensional score plots of LD1 and LD2. Box chart legend: *center line* = median; *center square* = mean; *edges* = 25th and 75th percentile; *whiskers* = SD. *Different letters* over box charts indicate statistically significant differences among groups ($p < 0.05$; one-way ANOVA and Tukey's multiple comparison test). ANOVA, analysis of variance; IR, infrared; LDA, Linear Discriminant Analysis; PC, principal component; PCA, Principal Component Analysis. Color images are available online.



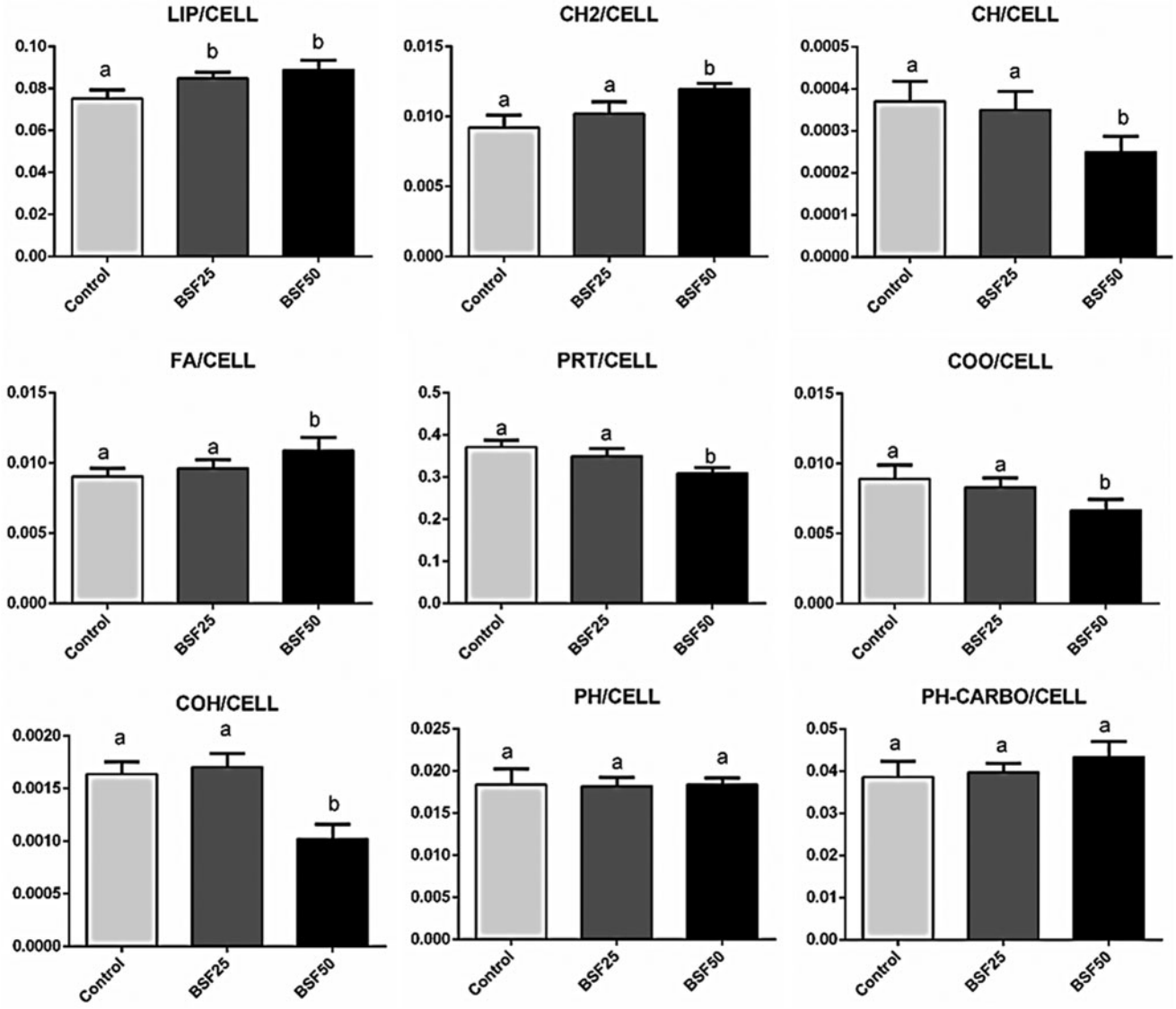


FIG. 10. Biochemical composition of the inner compartment of class IV oocytes from Control, BSF25, and BSF50 groups. Statistical analysis of the numerical variation of the following band area ratios: LIP/CELL, CH2/CELL, CH/CELL, FA/CELL, PRT/CELL, COO/CELL, COH/CELL, PH/CELL, and PH-CARBO/CELL. *Different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

of unsaturated ones (CH/CELL, $p < 0.05$) were detected. A significant decrease in protein amount (PRT/CELL, $p < 0.05$) and COO^- groups of amino acids (COO/CELL, $p < 0.05$) was observed only in BSF50. No significant changes were highlighted in phosphates (PH/CELL, $p > 0.05$) and carbohydrates (PH-CARBO/CELL, $p > 0.05$), while a decrement of glycosylated compounds (COH/CELL, $p < 0.05$) was found in BSF50.

Real-time PCR results

Real time PCR analysis performed on genes involved in the long-chain PUFA biosynthesis is presented in Figure 11. With regard to *elovl2* gene expression, a dietary BSF meal dose-dependent increase in gene expression was observed. BSF50 group showed a significant overexpression compared to Control ($p < 0.05$), while BSF25 did not show significant differences ($p > 0.05$) with respect to the other experimental groups. With regard to results about *elovl5* gene expression, the BSF50 group showed a significantly higher value compared to BSF25, while Control and BSF25 did not show significant differences between them. Finally, *fads* gene expression did not show significant differences among the experimental group ($p > 0.05$).

Discussion

In this study, the inclusion of BSF meal in the diets provided interesting results on zebrafish reproductive pathways. As reported in several reviews and papers on the use of insect meal in aquafeed,[62–67] insects are characterized by high

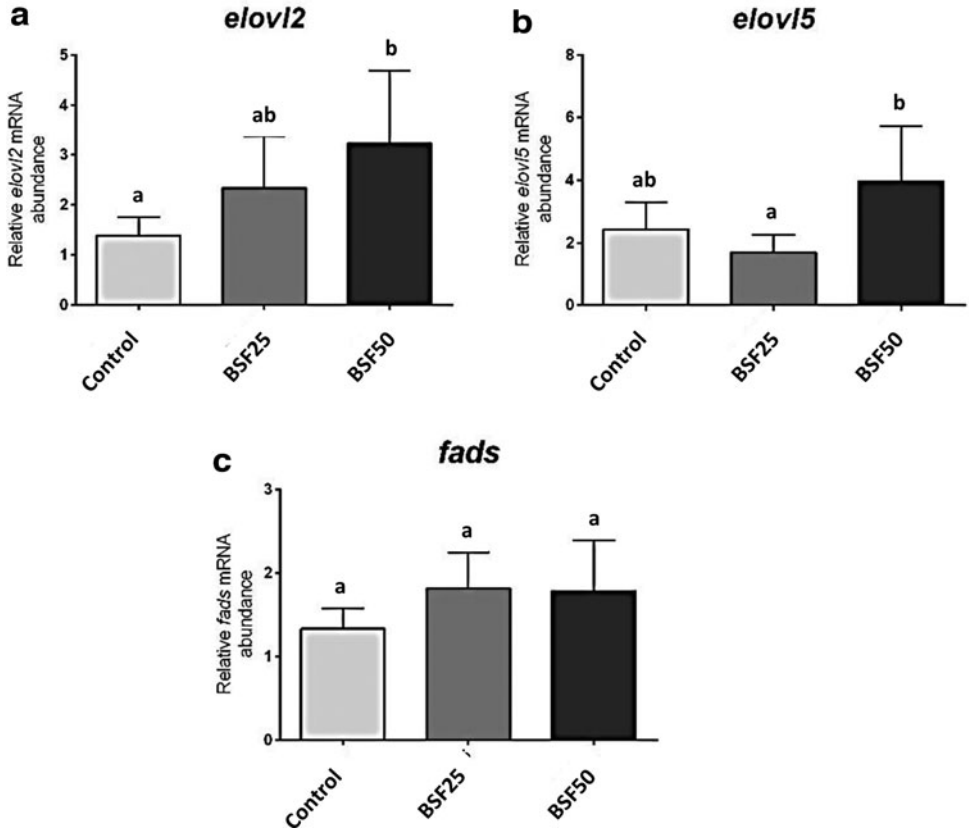


FIG. 11. Relative mRNA levels of genes involved in long-chain PUFA biosynthesis analyzed in zebrafish female's liver. **(a)** *elovl2*, **(b)** *elovl5*, **(c)** *fads*. *Different letters* indicate statistically significant differences among the experimental groups. Data are reported as mean ± SD ($n = 5$). Zebrafish fed diet based on FM (Control) and zebrafish fed diets with 25% (BSF25) or 50% (BSF50) replacement of FM with BSF meal. mRNA, messenger RNA.

quantity (60%–80%) and quality of proteins and are rich in essential amino acids. On the other hand, insects show an unbalanced fatty acid profile,[68,69] rich in SFAs rather than in polyunsaturated ones (PUFA), and this is known to affect fish growth and welfare, especially when high inclusion levels are used.[70–72] Recently, BSF larva fatty acid composition has been modified by varying the growth substrate.[72] Specifically, it has been demonstrated that culturing BSF larvae on an organic substrate enriched 10% *Schizochytrium* sp., significantly increased insects' PUFA content, and may represent a proper idea for further studies.[30]

It is well established that lipid nutrition is particularly important for fish,[60,73] since deficiencies in some of these molecules, such as arachidonic acid (ARA; 20:4n-6), EPA (20:5n-3), and DHA (22:6n-3), can cause a general decrease of fish health, poor growth, low feed efficiency, anemia and high mortality.[74] Presently, most of the studies focused on the role of PUFAs on fish body composition and fish physiological responses[75–77]; however, only few studies were focused on the effects of these molecules on fish reproduction.[78–80]

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

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

In this study, it was observed that the inclusion of BSF in the diets caused a parallel dietary reduction in PUFAs and an increase in SFAs. This unbalanced dietary lipid composition did not affect fish biometry and GSI (with the exception of a length reduction), while it deeply influenced fish FA composition, which reflected the same lipid composition shown by the tested diets.

Noteworthy, the same result was not evidenced in the FA composition of class IV oocytes and fertilized eggs. Specifically, similar amounts of SFA, MUFA, and PUFA were found in Control and BSF25 groups, and only in BSF50, a higher SFA and lower PUFA content were detected by gas mass chromatography. FTIR analysis confirmed these lipid changes (LIP/CELL, CH2/CELL, CH/CELL, and FA/CELL) in BSF50 group class IV oocytes. In particular, length and unsaturation rates of lipid aliphatic chains and amount of fatty acids were consistent with the fatty acid content and composition revealed by gas chromatography.

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

In many freshwater demersal spawners, eggs usually lack a prominent oil globule, and vitellogenin is thus the most important lipid carrier into growing oocytes.[86] In these fish, vitellogenin-derived yolk contains ~20% lipid by weight, and about 60%–80% of these lipids are represented by phospholipids, which are typically rich in PUFAs and are important membrane components for the developing embryo.[87] In contrast with the vitellogenin-associated lipids, oil globules mainly contain neutral lipids rich in MUFAs, which, in fishes, preferentially serve as metabolic energy reserve.[88] Oil droplets are extremely abundant in pelagic spawners, like marine perciforms, and can occupy up to half or more of the ooplasm volume.[89,90]

Because zebrafish is a demersal freshwater spawner, completely lacking oil globules, the accumulation of MUFAs in this species is not a prominent process and this could explain why MUFAs did not show significant differences among the experimental groups. However, histological analysis revealed a higher amount of lipid droplets in class IV oocytes from the BSF50 group, confirming the results obtained by FTIRM, which showed a higher amount of the total lipid fraction in this class.

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

In conclusion, the results obtained in this study showed that the substitution of FM with BSF meal up to 25% in female zebrafish diet did not affect reproductive performances, while an higher inclusion (50% with respect to FM) resulted in reproductive impairments, specifically in terms of number of spawned eggs. These results suggest that the application of new ingredients for aquafeed formulation should always be deeply investigated not only during the different life stages of fish but also during the reproductive event. Even if zebrafish, due to its high reproductive rate and to the abundant information that has recently become available from genomic sequencing, is one of the most studied experimental models in biomedical sciences, developmental biology, genetics, toxicology, and aquaculture, a standardized diet for this species is still lacking. A few specific zebrafish diets are now available in the market; however, it is not clear how much information has been collected by these companies on the physiological effects on fish, a main critical bottleneck that still remains. Nowadays, a great variety of feeds are usually used by the different research facilities to feed their zebrafish, possibly posing some limits on an easy comparison of the obtained research results. It is thus clear how, a standardized diet, sustained by a solid research on the physiological effects on fish, is now extremely necessary to the zebrafish community.

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