An endeavor to find starter feed alternatives and techniques for zebrafish first-feeding larvae: the effects on viability, morphometric traits, digestive enzymes, and expression of growth-related genes

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Abstract – Low and variable growth and survival rates (SR) of 6-10 days post-fertilization zebrafish larvae are a problem. This problem seems to be linked to starter feed characteristics. The present study is an attempt to find alternatives to address these requests. For this, larvae were fed fresh and lyophilized microalgae (*Chlorella*, *Scenedesmus*, and *Haematococcus*), egg yolk (YOLK), lyophilized *Artemia nauplii* (LAN) and a combination of them. The lowest SR was observed in algae-fed larvae. All died on day 11 showing an emaciated appearance, similar to starved larvae. The highest SR was observed in YOLK- and LAN-fed larvae that also showed an elongated anterior part of the body. Negative correlations of SR with *vegfaa* (vascular endothelial growth factor) and morphometric traits with *igf2a* (insulin-like growth factor) were also found and supported by changes at molecular level. The presence of algae in the digestive tract of the larvae and the observation of fecal droppings indicate that the algae have an appropriate size and are palatable. The increase in the digestive enzyme activity shows the larval effort to digest the algae. The fact that the algae-fed larvae died even before the larvae kept in starvation indicates the dramatic amount of energy that the larvae spent in microalgae digestion. Although both YOLK- and LAN-fed larvae had the highest SR, LAN group started to feed on *Artemia* nauplii sooner. This can be linked to the delayed growth in YOLK-fed larvae and an accelerated growth in the case of LAN-fed group. LAN is an expensive feed with negative effects on water quality whereas YOLK is a cheap and nutritionally balanced feed with fine granular texture that contributes to a larval SR similar to LAN without affecting water quality. In conclusion microalgae cannot be considered a suitable starter food for zebrafish whereas LAN and YOLK can be considered as good starter feeds.

Keywords.- Artemia; microalgae; *vegfaa* and *igf2a* gene expression; yolk; zebrafish larvae

1. Introduction

Zebrafish is a well known fish model used in a growing number of scientific disciplines[1,2] due to its rapid organogenesis and transparent body[3]. The ability to rear zebrafish from egg to adult in the laboratory is of paramount importance[4] although raising zebrafish from larvae to juveniles can be laborious, requiring frequent water exchanges, and a continuous culture of different live prey.[5]

The most challenging larval stage in this species, as in many other cultured fish[6] is the first-feeding phase. The main technical difficulty during this stage is to meet the nutritional demands of the larvae because feed items must be i) appropriate size, ii) easy to digest, iii) attractive, iv) available on a continuous basis to support metabolic demands,[7] and v) without producing excessive waste in the tanks.[1] In a way that the larvae receive a good and nutritive feed covering their nutritional demands while maintaining water quality.[4] The low[4] and variable[7,8] larval growth and survival of zebrafish during the first 5 days of exogenous feeding (days 6–10 post-fertilization [dpf]) is an obstacle. The problem affects not only the number of larvae needed, but also the time it takes to complete experiments, the overall cost of the research, and the quality of adult zebrafish used for breeding and other experiments.[4]

The starter food for first-feeding zebrafish larvae can be either live prey (e.g. paramecia,[9] salt-,[1,4,5,10,11] and fresh-water rotifers,[12] and dried rotifer sheet[3]) or commercially available powdered feed.[7,8]

Paramecium (180 μm length, 80 μm width and 50 μm diameter[9]) is the most common live food used for young zebrafish[13,14,15,16] although its culture is quite complicated, requires repeated subculture, filtration and sterilization steps,[4] and does not support the rapid growth of zebrafish juveniles.[7,17]

Saltwater rotifers (*Brachionus plicatilis*)[4] have small size, slow swimming behavior, they can be bioencapsulated,[6] improve larval survival up to 90%,[10] and are easier to culture than paramecia.[5] However, Nakayama *et al*.[3] observed that the use of rotifers lead to salt contamination of the culture medium and they cannot survive enough time in freshwater for frequent feeding of zebrafish larvae. Other authors indicated that they can tolerate salinities of 1–97 ppt[18] and can survive in nursery tanks for extended periods.[4] Freshwater rotifers (*Brachionus calyciflorus*) have also been considered as an alternative.[12] Regardless of the rotifer type, their maintenance and use for newly hatched zebrafish larvae is a challenge for small academic laboratories. To solve the problem Nakayama *et al*.[3] produced a dried rotifer sheet using cryptobiotic (*Bdelloid*) rotifers[19] as a simple and convenient live feed for rearing first-feeding zebrafish larvae.

Thus, rearing the live foods is one of the main problems in small scale low-cost laboratories that do not have access to the necessary funding, equipment, or personnel to maintain large scale systems usually employed in zebrafish husbandry.[5] To reduce this problem some powdered artificial feeds have been developed,[20,21] although growth and survival rate of fish fed with them are lower and more variable.[7] This has been attributed to insufficient/unsuitable nutritional profile, poor attractiveness and digestibility of the feeds.[6] The processed feeds also contaminate the culture medium[22] due to leaching of uneaten and decomposing feed particles.[6,23]

Since 1980, enormous efforts have been made to develop microdiets to replace live feed for marine fish larvae.[24] These microdiets have been also used in zebrafish facilities,[25,26] but they cannot completely replace live feeds for most marine species,[24] an important matter that should also be considered in the case of zebrafish.

Zebrafish are omnivorous, eating zooplankton, phytoplankton, insects, worms and small crustaceans[27] thus, several organisms can be potentially used as live food for rearing zebrafish first-feeding larvae in low-cost laboratories.

In the case of marine fish larvae (at very early developmental stages) one of the most used techniques is what it is called "green water technique" that consists in the use of microalgae (ALG) directly in the rearing tanks. Microalgae work as stimulants of the non-specific immune system in the larvae, control the microbial growth in the water and maintain water quality changing the nitrogenous wastes and CO_2 to O_2[28,29] and reducing N and P loads.[30] Although their role in larval nutrition is not clear,[31,32] they contribute in the settlement of a healthy intestinal microflora in fish larvae by preventing the development of opportunistic bacteria,[32,33] and reduce the light in the tanks increasing the contrast to reveal live preys).[30,34] In mariculture, ALG are also used to produce mass quantities of zooplankton (rotifers and *Artemia*) which serve as food for larval and early-juvenile stages of fish.[30,34] In Urmia University freshwater ALG are used to produce a freshwater zooplankton fairy shrimp *Branchinecta orientalis*,[35,36,37] as a potential live food for zebrafish.

ALG are generally used for live food culture, however, their effect as starter food for first-feeding fish larvae has not been evaluated yet. Thus, in the present study, in addition to ALG the suitability of three practical diets as an alternative to processed foods during the first 5 days of exogenous feeding is also evaluated. The potential of three freshwater algae (*Chlorella vulgaris* [*CV*], *Scenedesmus obliquus* [*SO*], and *Haematococcus pluvialis* [*HP*]), as live foods, together with lyophilized "*Artemia* nauplii " (LAN) and "egg yolk" (YOLK), as practical diets, to be used as a starter food of choice for rearing first-feeding zebrafish larvae in a low-cost laboratory are the main objectives.

For this, not only larvae were fed with alive microalgae –AALG- (i.e. alive *C. vulgaris* [ACV], *S. obliquus* [ASO], and *H. pluvialis* [AHP]), lyophilized microalgae –LALG- (i.e. lyophilized *C. vulgaris* [LCV], *S. obliquus* [LSO], and *H. pluvialis* [LHP]), LAN, and YOLK, but also a combination of AALG and LAN (i.e. ACV+LAN, ASO+LAN, and AHP+LAN), and a combination of LALG and LAN (LCV+LAN, LSO+LAN, and LHP+LAN). To tackle these objectives, these fourteen dietary treatments were compared taking into account embryo and larval survival, morphologic and morphometric changes in the larvae, and expression level of the genes *igf2a* (insulin-like growth factor) and *vegfaa* (vascular endothelial growth factor).

It has been cited that *igf*s signaling pathway regulate growth, development, metabolism, and longevity in a wide variety of animals.[38] The effects of nutritional status[39,40,41,42] and environmental parameters[43] on *igf*s expression have already been cited.

vegf gene family provide signals for *de novo* formation of blood vessels during embryogenesis and for the formation of new blood vessels from preexisting vessels during organogenesis.[44] The effects of diets supplemented with natural products on *vegf*s expression have already been cited.[45]

2. Material and methods

2.1. Animals, housing facility, and breeding

Broodfish (wild-type [AB line]) were purchased from a local supplier, and transferred to Urmia University where they were housed, maintained in a static system, and reproduced in a low-cost facility described in Samaee *et al*.[46]

2.2. Embryo/larvae culture

Glass beakers of 250 mL placed in a plastic container equipped with a heater were used for the incubation of eggs, and for larval culture. The beakers filled with 98 mL system water[46] were used to incubate 30 fertilized eggs at a temperature of 28 ± 0.5 ºC using a 14 h light:10 h dark photoperiod. The beakers were provided with a gentle and continuous aeration.

To prepare the system water, municipal (tap) water was dechlorinated, heated to 28 ºC, filtered through an active carbon filter, and then conditioned with 240 mg L^{-1} rock salt + 60 mg L^{-1} sea salt. Finally, the physiochemical parameters of the system water were tested and adjusted.

Each food group was arranged in four replicates (120 embryos per food group) and the newly hatched larvae reared in the same container. Zebrafish larvae from day 2 (48 hours post-fertilization [hpf]) to day 6 (days post fertilization [dpf]) use the reserves from the yolk sac for development and only 80% of the water was renewed once daily. From day 7 to 11 dpf larvae were fed two times per day and the water changed after each feeding (Fig. 1). The physico-chemical parameters of culture medium before and after adding food groups are presented in table 2.

Beakers were checked on a daily basis until 11 dpf: day 1 (0-24 hpf [hour post-fertilization]; embryogenesis), 2 (24-48 hpf; embryogenesis), 3 (48-72 hpf; hatching), 4 (72-96 hpf; passive feeding), 5 (96-120 hpf; passive feeding), 6 (120-144 hpf; onset of exogenous feeding), 7 (144-168 hpf; complete depletion of yolk), 8, 9, 10, 11 (7-11 feeding on starter food), and 12 (onset of feeding on *Artemia* nauplii). At each checking time, dead embryo/larvae were removed, the system water[46] exchanged, and viability parameters such as hatching and survival rates calculated.

2.3. Preparation of ALG culture and suspension

A disinfected 500-1000 mL Pyrex® bottle with a screw cap equipped with an aeration system and placed in a plastic container of 3-5 L was used for ALG culture. The container was half filled with tap water and equipped with a 150 W heater (25 °C), a thermometer, and a 6 W light. Nine hundred mL of distilled water (pH 8) together with 3 N BBM (Modified Bold's Basal Medium) and 100 mL of ALG stock were added and the culture kept at 25ºC under continuous aeration and light for 1 week. After 1 week culture 4-5 million ALG per mL were obtained and stored at 4ºC until use.

The bottle containing 1 L ALG culture was centrifuged (8S GMP, Sigma, Germany) at 6000 rpm for 5 min at room temperature (RT), 90-95% of supernatant discarded, and the ALG re-suspended in the remainder supernatant (50-100 mL) by shacking the bottle. The ALG concentrated suspension was decanted into a 50 mL falcon tube, centrifuged at 5000 rpm (5 min at RT), the supernatant discarded and the falcon tube containing precipitated ALG placed in a freezer (-20 ºC) overnight. The cap of the falcon was removed, the falcon placed in a freeze-dryer (SBPE, Zistfarayand Tajhiz Sahand, Iran) overnight, the LALG ground, and stored at 4 ºC until use. For feeding zebrafish larvae 0.1 g of this LALG powder were dissolved in 30 mL water and mixed well. The food group was performed in 4 replicates (with a total of 30 larvae per replicate). From days 7 to 11 (first feeding larvae) 2 mL of the suspension was added to beakers, left for 1 h, two times per day, and the water changed after each feeding. Lyophilized *CV* particulate size in water was: 7-133 µm; *SO*: width = 3-10 and length = 10-30 µm; and *HP*: diameter = 20-80 µm.

In the case of feeding with AALG 30 mL of ALG culture were decanted into a 50 mL falcon tube, centrifuged at 5000-6000 rpm (5 min at RT), and the supernatant discarded. 30 mL system water was then added, falcon shaken, centrifuged at 5000-6000 rpm (5 min at RT), and the supernatant discarded, repeating several times this procedure. Finally, a known mL of water added to washed ALG to achieve a number of 4,500,000 ALG per mL, the falcon tube shaken several times and the suspension stored at 4 ºC till use. Four different methods (Fig. 1) were used to feed the larvae with AALG. Each method was performed in 4 replicates (with a total of 30 embryo/larvae per replicate):

Method 1: Embryos (0-2 dpf) and passive feeding larvae (3-6 dpf) were cultured in clear water, 80% of water changed daily. From days 7 to 11 (first feeding larvae) 2 mL of the ALG suspension was added to the glass beakers (containing 98 mL medium [a final ALG density of 90000 ALG per mL] in which 30 larvae had been cultured) and left for 1 h for larval feeding. This was done twice daily and the medium changed after each feeding.

Method 2: From days 7 to 11 larvae were cultured in green water (90000 ALG per mL), ALG precipitates removed (3 times daily) followed by checking ALG density and adjusting to 90000 ALG per mL.

Method 3: Fertilized eggs were cultured in green water, from hatching to the day 11, ALG precipitates removed (3 times daily) followed by checking ALG density and adjusting to 90000 ALG per mL.

Method 4: Fertilized eggs were cultured in green water (90000 ALG per mL), on day 6 the green water replaced with system water. From days 7 to 11 post-fertilization 2 mL of the ALG suspension was added to the glass beakers (a final ALG density of 90000 ALG per mL) and left for 1 h. This was done twice daily, medium changed after each feeding.

2.4. Fatty acid (FA) composition of ALG

For this, 200 mg sample of microalgae were transferred to a 35 mL glass tube with a Teflon lined screw cap, 1 mL of a freshly prepared methanol-sulfuric acid mixture (2.5% H_2SO_4 in CH_3OH) added, gradually heated, and shaken every 10 min, in a water bath to 80 °C, incubated for 1 h, cooled to RT, 500 µL hexane and 1.5 mL NaCl (0.9%) added, shaken vigorously, centrifuged (at 4000 rpm for 5 min), and supernatant (1 µL) subjected to gas-liquid chromatography (GC). The determination of FA composition was done as described by Samaee *et al*.[46]

2.5. Antioxidant profile of ALG

Dried ALG was used to determine the antioxidant composition. To obtain dried ALG, the glass container with 1 L microalgae culture was centrifuged at 6000 rpm for 5 min, 90-95% of supernatant discarded, and the precipitated ALG re-suspended in the remainder supernatant (50-100 mL) by shacking the container. The concentrated ALG suspension was decanted into a Petri dish, transferred to an incubator that had been adjusted to ≥ 50 ºC overnight and the dried ALG collected into a pre-weighed 15 mL falcon tube, labeled and stored at 4 °C until biochemical analyses. For the analysis, 0.1 g ALG was homogenized in 1 mL acetonitrile, sonicated for 10 min, centrifuged at 2500 rpm for 3 min at RT, and the supernatant stored at -20ºC until use. 0.5 μL of this supernatant were used for GC-mass analysis using an Agilent 7890 A gas chromatograph coupled to a 5975A mass spectrometer using a HP-5 MS capillary column (5% Phenyl Methylpolysiloxane, 30 m length, 0.25 mm i.d., 0.25 μm film thickness). The oven temperature was programmed as follows: 3 min at 80 °C, subsequently 8 °C min^{-1} to 180 °C, held for 10 min at 180 °C. Helium was used as carrier gas at a flow rate of 1 mL min^{-1} and the Electron-impact (EI) was 70 eV. The injector was set in a split mode (split ratio of 1:500) using a mass range acquisition from 40 to 500 m/z. Antioxidant constituents were identified by using the calculated linear retention indices and mass spectra with those reported in the NIST 05 and Wily 07.

2.6. Preparation of AN and LAN suspension

Artemia nauplii (AN) suspension was prepared as in Samaee *et al*.[46]. The *Artemia* cysts (AC) (*Artemia franciscana*, strain VC) used in the current study were provided by Can Tho University, Vietnam. *Artemia* cysts hatched (2 g AC per L) after being incubated during 24h in filtered tap water with 33% rock salt, 8.5 pH, vigorous and continuous aeration, 28ºC water temperature and continuous light. Hatching rate (AN%) determined, newly hatched *Artemia* nauplii (instar-I; width = ~ 225 and length = ~ 500) harvested and the suspension prepared washing a known number of AN into a beaker with a different dispersant (system water, 33% salt water, or collected culture medium) depending on the goal of research. Larvae after 11 dpf were fed 2 times per day with a concentrated AN suspension (1270 AN mL^{-1}): 1 h after turning the light on and 7 h later.

To prepare LAN suspension, AN were collected in a 100 µm mesh-basket, washed with distilled water and transferred to a falcon tube before being freeze-dried as in the case of LALG (see section 2.3). For feeding zebrafish larvae 0.1 g of this LAN powder were dissolved in 30 mL water and mixed well. The food group was performed in 4 replicates (with a total of 30 larvae per replicate). From days 7 to 11 dpf (first feeding larvae) 2 mL of the suspension was added to beakers used for larval culture and left for 1 h, two times per day, and the water changed after each feeding.

2.7. Larval feeding with a combination of ALG (alive and lyophilized) and LAN

From days 7 to 11 dpf (first feeding larvae) 1 mL of the AALG (i.e. ACV, ASO, and AHP) or LALG (i.e. LCV, LSO, and LHP) suspension (see the section 2.3) and 1 mL ALN suspension (as combined diet denoted as AALG+LAN [i.e. ACV+LAN, ASO+LAN, and AHP+LAN] and LALG+LAN [i.e LCV+LAN, LSO+LAN, and LHP+LAN]) was added to beakers, left for 1 h, two times per day, and the water changed after each feeding. Each feeding group was performed in 4 replicates (with a total of 30 larvae per replicate).

2.8. Preparation of YOLK suspension and larvae feeding

A small piece (chickpea size) of a hard-boiled egg was put in a mesh, wrapped, soaked in 50 mL water, and pressed until obtain a cloudy water. From days 7 to 11 dpf (first feeding larvae) 2 mL of the freshly prepared YOLK suspension were added to beakers and left for 1 h. This was done twice daily and water changed after each feeding. The food group was performed in 4 replicates (with a total of 30 larvae per replicate).

2.9. Larval sampling

Zebrafish larvae were sampled at different ages (days post fertilization) to assess growth, digestive enzyme activity and gene expression. Sampled larvae were transferred to a Petri dish with 20 mL sterilized phosphate buffer solution (PBS; 1 tablet in 200 mL dH_2O, Sigma-Aldrich) where they swim and washed. Ten (for gene expression analysis) or twenty (for enzymes activity) larvae taken from each dietary group, pooled in 3 mL cryo-tube, labeled, and PBS removed, 1 mL PBS added for a final washing of the larvae, PBS totally removed, snap-frozen immediately in liquid nitrogen, and stored at -80°C till use.

For morphometric analyses eight larvae were randomly taken from each dietary group and fixed in 10% neutral buffered formalin (10 mL of 37% formaldehyde, 0.9 g sodium chloride, and 100 mL water[47]) for 24 h and then stored in 4 ºC.

2.10. Determination of enzyme activity

To prepare crude enzyme extract (CEE) 100 μL sodium phosphate buffer (0.025 M, pH 7.2) was added to sample (20 frozen larvae, see section 2.13) (1:3), homogenized with a pellet pestle, centrifuged at 10000 g for 20 min at RT (Bekman Coulter centrifuge, Allegra 2IR, Germany), the supernatant collected, divided in four parts, and stored at -80 °C. The samples were analyzed in triplicate (biological replicates) and each of them examined in triplicate (methodological replicates).

Total soluble protein of larvae was measured by Bradford[48] method and the results presented as mg per mL.

Alpha-amylase (E.C.3.2.1.1) activity was determined according to Worthington[49] using starch as substrate and using Maltose (Merck, Darmstadt, Germany; 0 - 5 μmol mL^{-1} deionized water) to build the standard curve. The α-amylase specific activity was defined as 1 μmol maltose produced per min per mg protein at 25 °C.

Bile salt-activated lipase (E.C.3.1.1) was determined using nitrophenyl myristate as substrate according to Iijima *et al*.[50]. The lipase specific activity was defined as 1 μmol of n-nitrophenol released per min per mg protein.

Total alkaline proteases (TAP) were assayed by the azocasein hydrolyses method described by Garcia-Carreño and Haard[51]. The unit alkaline protease specific activity was expresses as the change in absorbance at 440 nm per min per mg of protein.

2.11. Hatching and larval survival rate

In the time of water change, dead embryo/larvae were removed and hatching and survival rate was calculated. The hatching rate (HR) was calculated as the ratio of hatched embryos divided by the total number of cultured embryos × 100 at 1-3 dpf. SR was estimated for zebrafish larvae from 7 dpf (complete depletion of yolk) to 11 dpf (the time when zebrafish larvae start to feed on AN). SR was calculated as the ratio of alive embryo/larvae to total number of cultured embryos × 100.

2.12. Larval morphometric characteristics (MCs)

Photomicrographs of the fixed larvae were taken at 11 dpf using a stereomicroscope (Zeiss, Germany) equipped with a digital camera (Carl Zeiss Inc.). MCs were measured on digital images using Image J 1.48 program. Five MCs were recorded and then used to calculate 15 morphometric ratios (Fig. 2).

2.13. Gene expression analysis

Ten larvae from each group were sampled, washed in phosphate buffer solution (PBS; 1 tablet in 200 mL autoclaved dH_2O, P4417, Sigma-Aldrich), pooled in a 3 mL cryotube, considered as one sample, labeled, snap frozen in liquid nitrogen and stored at -80 ºC until use. Total RNA was extracted using a Bio FACT™ Kit (RP101- 050, Daejeo, Korea) following the manufacturers' instructions, eluted in 20 μL RNase-free water, its quality/quantity determined by spectrometry (NanoDrop-Thermo 2000C; Thermo Fisher Scientific, Wilmington, DE, USA) of total RNA solution, and finally stored at -80°C. Total RNA (10 ng) was reverse transcribed into cDNA using a BioFACT™ Kit (BR441-096, Daejeo, Korea) in a 20 μL reaction volume by a thermal cycler (model, PEQLAB, Germany), cDNA quantity evaluated by spectrometry, and stored at -20 °C.

The two target genes (*vegfaa* and *igf2a*) and a housekeeping gene (*β-actin*,[52] used as internal standard for the target genes) were considered for gene expression analysis. *β-actin* did not change among different food groups. The required primers were designed with Primer Express Software (Applied Biosystems) using identical parameters to generate amplicons of similar size. The primers were synthesized by Metabion International AG (Germany) (Table 1).

Quantitative real-time PCR was performed using the StepOne Plus™ system (Applied Biosystems, Foster City, CA, USA). Each reaction contained 0.5 μL diluted cDNA, 0.05 μL forward (10 μM) and 0.05 μL reverse (10 μM) primers, and 10 μL SYBR Green PCR Master Mix to a final volume of 20 μL. Amplification followed the PCR cycle condition: 95 ºC for 10 min, followed by 40 cycles of 15 s at 95 ºC and 1 min at 61 ºC. Each food group was analyzed in triplicate (biological replicates) and each sample in triplicate (methodological replicates). A non-template control was performed to ensure that only one PCR product amplified and the stock solutions were not contaminated. Cycle conditions and amounts of templates were optimized for each primer set in pilot experiments to ensure that amplification was terminated within the linear phase.

A melting curve was also performed to ensure the specificity of PCR amplification. The melt curve protocol was 15 s at 95 °C for one time and then 10 s each at 0.3 °C increments between 60 °C for 1 min and 95 °C for 10 min. Data collection was enabled at each increment of the melt curve. The amplification efficiency, specificity of primers, and amount of cDNA/sample were evaluated by the standard curve method. Primer pairs were deemed to be acceptable for *vegfaa* and *igf2a* expression analysis if they generated standard curves with an r^2 value above 0.98, there was consistency among replicates, and the primer amplification efficiency was 85–110%.

The *vegfaa* and *igf2a* RNA expression levels are presented as cycle threshold values. The relative expression of genes was calculated by the $2^{-\Delta\Delta CT}$ method[53] using *β-actin* according to the formula: i) $\Delta CT_{Control} = CT_{target\ gene} - CT_{housekeeping\ gene}$, ii) $\Delta CT_{Exprimental} = CT_{target\ gene} - CT_{house\ keeping\ gene}$, iii) $\Delta\Delta CT = \Delta CT_{Exprimental} - \Delta CT_{Control}$, and iv) fold change: $2^{-\Delta\Delta CT}$.

2.14. Statistical analysis

Data normality was tested by the Anderson-Darling method. Univariate analysis of variance (ANOVA; followed by Duncan's multiple range post hoc test) was used to test the differences among food groups for HR, SR, MCs, enzyme activity, and gene expression level. A *p*-value of 0.05 was accepted for statistical significance. Simple regression models were formulated to characterize endpoints that are correlated to HR and SR. A *p*-value of < 0.002 was accepted for determining the level of significance for the regression analysis; considered to be the statistical significance threshold after applying the Bonferroni's adjustment for the critical value of $p < 0.05$ to minimize the chance of type I statistical error. All statistical analyses were performed using IBM SPSS (version 20; SPSS Inc., Chicago, IL, USA), and Excel 2010 (Microsoft Corporation, Redmond, WA, USA).

3. Results

Tables 3 show the fatty acid composition of the microalgae used in the current study. Significant differences were found among the three freshwater algae (i.e. *C. vulgaris*, *S. obliquus*, and *H. pluvialis*) in their fatty acid (Table 3) and antioxidant (data not shown) composition.

The embryos cultured in water using the methods 1 and 2, hatched on day 3 (72 hpf, 54-66%) (Table 4, rows 3, 6, and 9), while those cultured in AALG suspensions using the methods 3 and 4 described in section 2.3 (Table 4, rows 2, 4, and 8) began to hatch at day 2 (36 hpf, 54-92%), leading to a significant difference between the two groups in HR on day 2 (24-48 hpf) and 3 (48-72 hpf).

The method of culture did not have any effect on SR at different larval stages, therefore method 1 was considered the method of choice in the study to evaluate not only ALG-based starter foods (ALG and ALG+LAN) but also other food groups (YOLK and LAN).

No significant variation were found in survival rate (Table 5, rows 2-6) and MCs (Table 5, rows 8-28) among the larvae fed AALG (ACV, ASO, and AHP), LALG (LCV, LSO, and LHP), and between AALG- and LALG-fed larvae, thus the ALG-based groups were considered as a single group.

The same results, no significant differences, were found in SR (Table 6, rows 2-6), and MCs (Table 6, rows 8-28) among A_{CV}+LAN-, A_{SO}-+LAN-, and A_{HP}+LAN-fed, among L_{CV}+LAN-, L_{SO}+LAN-, and L+LAN-fed, and between AALG+LAN- and LALG+LAN-fed larvae, therefore the ALG+LAN-based groups were considered as a single group, as well.

Regarding larval survival rate it was observed that on day 7 the starved (STA) larvae had the lowest SR (Fig 2a) whereas a significant decrease was detected in ALG- and ALG+LAN-fed larvae at 8 dpf (28.6% and 2.4%, respectively; Fig 2b), 9 dpf (49.1% and 6.4%; Fig 2c), and 10 dpf (49.4% and 30.8%; Fig 2d). On days 8 (Fig 2b) and 9 (Fig 2c) the lowest SR was related to ALG-fed larvae followed by STA while at 10 dpf the lowest SR was observed in ALG- and ALG+LAN-fed larvae (Fig 2d). At 11 dpf all the ALG-fed larvae died and SR was even more reduced in ALG+LAN-fed, and STA larvae (Fig 2e).

The enzyme activity was measured to evaluate the response of larval digestive tract to feeding on ALG at different developmental stages. For this, 6 groups of larvae were used for enzyme activity determination: (1) LAN-fed larvae that showed the highest survival rate at 11 dpf, the onset of feeding on *Artemia* nauplii, (2) ALG-fed at 8 dpf (complete absorption of yolk and shift on exogenous feeding,[54] 9-10 dpf, and 11 dpf, (3) larvae before active feeding (BF) at 6 dpf (open digestive tract with enzymes secretion[54]), and (4) STA at 11 dpf.

The highest and statistically significant ($p < 0.05$) lipase activity (U mg^{-1} protein) was observed in ALG-fed larvae at 9-10 dpf, higher than ALG-fed larvae at 8 dpf (2 times) and 11 dpf (10 times) and the other groups (10 times). ALG- and LAN-fed larvae at 11 dpf had a non-significantly ($p > 0.05$) higher (3 times) lipase activity than the BF larvae at 6 dpf and STA larvae at 11 dpf (Fig. 5a).

The highest amylase activity was observed in the ALG-fed larvae at 9-10 dpf (4 and 11 times higher than ALG-fed larvae at 8 and 11dpf, respectively). No significant differences were observed among STA, ALG-fed, and LAN-fed larvae at 11 dpf) concerning amylase activity, being lowest in BF at 6 dpf (Fig 4b).

Alkaline protease activity (U mg^{-1} protein) was only detected in LAN-fed larvae at 11 dpf (Fig 4c).

Significant variations among the groups (YOLK-, LAN-, ALG-, ALG+LAN-fed, and STA larvae) were observed concerning MCs at 11 dpf (Table 7). Thus, differences were found in the length of the anterior part of the body (APB, Fig 5a) and the ratios body length/head length (BL/HL, Fig 5c), body length/anterior part of the body (BL/APB, Fig. 6e), anterior part of the body/posterior part of the body (APB/PoPB, Fig 5g), and posterior part of the body/head length (PoPB/HL, Fig 5i). These five MCs were significantly correlated to SR at 11 dpf (Fig 5b, d, f, h, and j) in the form of linear (Fig 5b, f, and h) and quadratic (Fig 5d, and j) functions while other ratios were not significantly associated to SR.

The expression level of *vegfaa* was down-regulated (by 0.47-fold) in the STA larvae relative to control group whereas it increased in the other groups. A significant variation among the groups in terms of *vegfaa* expression (Fig. 7a). The highest expression level was observed in ALG (4.25 fold) followed by YOLK (2.18), ALG+LAN (1.87), and LAN (1.10) food groups.

An increased expression level of *igf2a* was observed in all food groups relative to the control (10-176 fold). A remarkable variation among the larvae fed on different food groups concerning *igf2a* expression level (Fig. 7c) in a way that the highest *igf2a* expression level was observed in ALG (175.98 fold) followed by ALG+LAN (162.39), YOLK (152.38), LAN (102.66), and SAT (9.59).

SR at 11 dpf was significantly correlated to *vegfaa* expression in the form of a linear function (Fig. 7b) whereas no correlation was found with *igf2a*. A significant association was observed between *igf2a* and MCs at 11 dpf (APB [Fig. 7d], BL/HL [Fig. 7e], BL/APB [Fig. 7f], and PoPB/HL [Fig 6g]) in the form of quadratic functions whereas such relationships could not be found with *igf2a*.

4. Discussion

Although no significant differences were found in the hatching rate (HR , Table 4) of the embryos cultured with different microalgae, an earlier hatching at days 2 and 3 (72-96 hpf) was observed, when compared to the eggs incubated with water. Early hatching has been cited as an important stress response of fish larvae[55] and similar changes in hatching events have been cited in embryos exposed to nanoparticles[56,57,58] and crude oil extracts.[59] Hatching is a consequence of the production of hatching enzymes and embryo movements[60,61]. In the case of nanoparticles, they tend to adhere to the surface of the egg chorion, blocking the pores that facilitate oxygen exchange and waste elimination[62,63,64] whereas crude oil extracts cause an increase in respiration and gill ventilation rates in the embryo, that facilitates an early rupture and release of hatching enzymes from the glands.[59] In the present study the change in hatching time (Table 4) can be attributed to the effect of organic acids produced by the algae on egg shell.

The early hatched embryos obtained were smaller in size and with larger yolk sacs relative to body size, indicating that the early hatching is not a result of a faster embryogenesis but a consequence of premature hatching.[57,58] However, this early hatching did not affect larval SR at different developmental stages (Tables 5 and 6).

The lowest survival rate was observed in ALG-fed larvae (Fig. 3 b-e), all the larvae died on day 11 post fertilization (Fig. 3e). The presence of ALG in the digestive tract of the ALG-fed larvae (Fig. 4a), as well as the observation of larvae releasing fecal droppings (Fig. 4a), confirms that they have fed actively on ALG indicating that ALG are appropriate in size and attractive for zebrafish first-feeding larvae, so the mortality of the larvae (Fig. 3e) cannot be attributed to the failure in feeding on ALG.

The dead larvae fed on ALG had an emaciated appearance similar to that of STA larvae (Fig. 4b). In the ALG-fed larvae the activity of digestive enzymes such as amylase (Fig. 5a) and lipase (Fig. 5b) started to increase on day 8, reached a maximum level through days 9-10, and decreased, to a level similar to that of STA larvae, on day 11 the day in which all the emaciated ALG-fed larvae died. The significant increase in the activity of the digestive enzymes in ALG-fed larvae compared to STA larvae shows a remarkable endeavor in ALG-fed larvae to digest the ingested ALG. The emaciation of the dead ALG-fed larvae can be related to the failure in ALG digestion. Zebrafish is an omnivorous species with both cellulose-degrading and protease-producing gut microbiome.[65] However it might be the case that at early larval stages the cellulolytic enzyme-producing bacterial community has not been established in the gastrointestinal tract of zebrafish. Yokoe and Yasumasu[66] mentioned that fish does not posses endogenous cellulose and therefore cellulose digestion depends on the exogenous cellulose produced by microbiota.

The activity of amylase (Fig. 5a) and lipase (Fig. 5b) in ALG-fed larvae was also higher than LAN-fed larvae whereas the alkaline protease activity detected in the LAN-fed larvae (Fig. 5c) was not observed in ALG-fed larvae. The qualitative and quantitative variation in digestive enzymes profile in fish have already been asserted to feed type, quality (digestibility), and nutritional status.[67] In the young carps the activity of digestive enzymes showed an adaptation to a dietary change within a week.[68]

All the ALG-fed larvae died on day 11 post fertilization, earlier than STA larvae (Fig 2e). This accelerated mortality compared to STA larvae can be associated to the dramatic amount of energy spent by ALG-fed larvae to digest ALG.

In order to understand the early death of ALG-fed larvae several factors of ALG-related products were analysed. ALG excretions were excluded as one of the causes of early death of the larvae having in mind the frequent washing of harvested ALG (see section 2.3). Other factors such as ALG-related endotoxins such as larvicides, cytotoxins, nematicides, pesticides, and antioxidants, were analysed (data not shown) without finding any difference in larval survival (Table 5) fed on ALG containing different phytochemical profile. Thus, the early death of zebrafish larvae cannot be attributed to ALG related products. Furthermore, distilled water was used to prepare ALG medium to avoid any possibility of water contamination (i.e heavy metals or other toxic products)

The premature death of the ALG-fed larvae might also be related to the nutritional composition of the ALG. However, the amounts of essential polyunsaturated fatty acids (PUFAs) such as 18:2*n*-6 (LA) and 18:3*n*-3 (ALA) in ALG (Table 3, rows 15 and 17, respectively) is higher than that of *Artemia*. Zebrafish can convert these two FAs to longer and more unsaturated homologues (high-unsaturated FA [HUFA]) such as 20:4n-6, 20:5*n*-3, and 22:6*n*-3.[69,70,71] On the other hand, larvae fed on *Spirulina* with a FA profile [Table 3, column 7] similar to that of *CV*, *SO*, and *HP* [Table 3, columns 4, 5, and 6, respectively] and a higher protein content (73.10% ± 1.04) had a SR (data not shown) comparable to that of *CV*-, *SO*-, and *HP*-fed larvae. Considering all this information, the low SR at different dpf [Fig. 3b-e] in the ALG-fed larvae compared to STA larvae (Fig. 3), the malnutrition-related early death should be rejected.

Microalgae species used in aquaculture are not equally successful in supporting the growth and survival of a particular fish. Suitable ALG species have been selected on the basis of their mass-culture potential, cell size, digestibility, and overall food value for a feeding animal.[34] In our laboratory the three freshwater ALG (*CV*, *SO*, and *HP*) are used to feed *B. orientalis*, and their mass-culture protocols are available.[35,36,37] The presence of ALG in the digestive tract of the larvae (Fig. 4ab) indicates that they have an appropriate size and are attractive to the larvae. The high essential FA content in *CV*, *SO*, *HP*, and *SP* compared to AN (Table 3), as well as the high protein content of *SP* (73.10%) also show the overall good nutritional value of ALG. Thus, the low survival rate and early death of zebrafish larvae in the present study can only be attributed to the low or nil digestibility of ALG (*CV*, *SO*, *HP*, and *SP*) in first-feeding larvae.

The highest larval survival rate (> 90%) was obtained in LAN- and YOLK-fed groups at all days post fertilization (Fig. 3). No significant differences between LAN- and YOLK-fed larvae were detected in SR and no differences could be found in SR at day 7 to 11. These results highlight the potential of these two simple feeds to support zebrafish early larval success. In at least one early report by Carvalho *et al*.[7], AN have been shown to support high growth and survival as a first-feeding item for zebrafish, but in practice this is difficult because the nauplii are often too large and too fast to be captured and ingested by first-feeding zebrafish larvae.[23] The results of the present study show that LAN can be used as an alternative to address these shortcomings. Takahashi[72] used a combination of YOLK (suspended daily in the rearing water) and live freshwater copepods to raise zebrafish larvae in an early growth period of about 10 days after hatching.

Adding LAN (50%) to ALG-fed larvae led to an increase in the SR of this ALG+LAN-fed larvae compared to ALG-fed group although a significant decline in SR of ALG-LAN-fed larvae from day 7 to 11 was observed (Fig. 3). In the case of ALG-fed larvae, all the larvae died on day 11, while ALG+LAN-fed larvae had a SR of 28%. Furukawa and Ogasawara[73] reported that the protein digestibility of a diet with different cellulose levels did not show differences although the growth rate decreased in accordance with the increase in cellulose content contrary to the results of our study where the presence of ALG in the ALG+LAN-fed larvae led to a significant decline in SR compared to LAN-fed larvae.

The results obtained in the present study show food-dependent changes in the morphometric characteristics (MCs) of the larvae (Table 7). Morphometric based assays are considered relevant to study fish larval growth and developmental response to several agents,[74] such as nanomaterials,[58,75] drugs,[76] and endocrine disrupting chemicals.[77] MCs are used to assess the impact on larval growth and development when they are exposed to concentrations well below those that change larval survival or produce an increase in malformations (Prof. Dr. Jennifer L. Freeman, Purdue University; Pes. Com). Thus, the significant variations of MCs detected among larvae fed on different types of feed (Table 7) evidence that MCs can be efficiently used to characterize diet-induced changes in larval growth and development.

Regarding the MCs (Fig. 6) the largest anterior part of the body was observed in larvae from LAN and YOLK groups followed by STA, and ALG+LAN- and ALG-fed ones. Fish growth delay is often related to starvation or malnutrition[78,79] and these can be the reasons of the smaller size of the anterior part of the body observed in STA, ALG+LAN- and ALG-fed larvae. The size of the anterior part of body is a determinant factor for larvae to shift on *Artemia* nauplii in zebrafish leaving behind a critical larval stage.

No significant differences in SR could be found between LAN- and YOLK-fed larvae although LAN group started to feed on *Artemia* nauplii sooner. This can be attributed to the bigger size of the anterior part of body in the LAN-fed larvae compared to YOLK-fed larvae. In other words, feeding with LAN accelerated larval growth. The high energy-protein ratio of YOLK as a diet for very young fish could result in an inadequate protein intake necessary for maximal growth[80] explaining the smaller anterior part of body in YOLK-fed larvae. On the other hand, the presence of digestive enzymes in *Artemia* nauplii has led to the speculation,[81] that these exogenous enzymes can play a significant role in the breakdown of the nauplii in the digestive tract of larvae. Thus, the relatively low levels of digestive enzymes in first-feeding larvae and subsequently the decrease in the digestion energy-expenditure can contribute to the bigger size of the anterior part of body in LAN-fed larvae.

Bahary *et al*.[82] isolated a duplicated *vegfa* locus in zebrafish (denoted as *vegfaa* and *vegfab*). Transfection of native zebrafish *vegfa* constructs into mammalian cell lines showed that *vegfaa* is secreted, but *vegfab* is not, even though a well-defined signal peptide is present. In the current study a nutritionally-induced change in *vegfaa* expression level in zebrafish larvae at 11 dpf relative to control was observed. The magnitude of the significant variation in *vegfaa* expression level was food-dependent (Fig. 7a). Such responses have also been cited by Chakraborty *et al*.[83] when the zebrafish larvae had been exposed to caffeine, norfloxacin, and nimesulide.

The *vegfa* gene is a highly specific vascular endothelial cell mitogen.[84] It is the strongest pro-angiogenic factor in the *vegf* family and it is expressed in every tissue.[85,86] The *vegf* gene family provides signals required for vasculogenesis (*de novo* formation of blood vessels) during embryogenesis and for angiogenesis (formation of new blood vessels from preexisting vessels) during organogenesis.[44] In the present study a significant association between SR at 11 dpf and *vegfaa* expression was observed (Fig. 7b) being SR variations (Fig. 3) supported by changes at molecular levels (Fig. 7a).

*igf*s are evolutionarily ancient growth factors present in all vertebrates.[87] It is comprised of four exons spanning 5981 bp on chromosome 7.[88] The *igf2a* acts through a conserved signaling pathway that regulates growth, development, metabolism, and longevity in a wide variety of animals.[37] In zebrafish, mRNA expression of the *igf2a* is observed in embryonic.[87,89] The nutritionally induced changes in *igf2a* expression level in zebrafish larvae at 11 dpf in the present study (Fig. 7c) is consistent with early studies such as those by Chauvigne *et al*.[90] who cited an increase *igf2* expression in refed juvenile rainbow trout after prolonged fasting periods, Thissen *et al.*[42] that observed a glucose-induced rise in *igf2* mRNA by stimulation of gene transcription and increased transcript stability, Soliman *et al*.[39] cited reduced serum *igf2* concentrations in chronically malnourished children and Straus and Takemoto[40] a decreased *igf2* mRNA in amino acid-deprived rat liver from alterations in posttranscriptional regulatory mechanisms. Thus, nutritional status is an important determining factor in the expression and secretion of metabolic hormones such as insulin family peptides.[38] In our study, MCs were significantly correlated to *igf2a* expression level in zebrafish larvae at 11 dpf (Fig. 7d-g). The *igf2a* function in the dorsal midline tissue during segmentation (defined by somitogenesis, elongation of the anteriorposterior axis, and neurulation – Kimmel *et al*.[91] and during early development[88]. The *igf2* mediates notochord formation and nephron development.[92] The observed effects are significant because notochord development is a highly conserved aspect of chordate development: the notochord, a transient mesoderm structure, secretes inductive cell signaling factors (e.g. Hedgehog proteins) that promote patterning of adjacent tissues, including the neural tube and somites.[88] It is clearly evident that *igf2* retains importance during post-hatching development in teleosts, possibly as a local paracrine and/or autocrine regulator of tissue growth.[38] Thus, in the present study a significant association was observed between MCs and *igf2a* expression (Fig. 7d-g) and the biometric variations (Fig. 6a, c, e, g, i) were supported by changes at molecular levels (Fig. 7c). In the studies of Zarantoniello *et al*.[93] in which zebrafish was fed on black soldier fly (BSF)-based diets such biometric variations were not correlated with *igf2a* expression level.

ALG were not digestible for zebrafish early larvae and the enzymatic content of LAN[81] make them more digestible than YOLK. In other words, digestibility was different depending of the food used. The lowest *vegfaa* and *igf2a* expression was observed in zebrafish larvae fed on more digestible feeds (Fig. 7a and c), thus, it seems that there is a negative association between feed digestibility and *vegfaa* and *igf2a* expression level.

Carvalho *et al*.[7] noted that zebrafish larvae are routinely reared on paramecia during the first 9 days of exogenous feeding, followed by a combination of paramecia and AN until day 21. Varga[9] cited paramecium from days 5-10, Zeigler AP 100 (a food that is not available) from day 11-12, and AN thereafter. The above mentioned feeding strategies are expensive, labour intensive, and unpredictable.[7] Feeding of zebrafish larvae with LAN suspension from day 5(6) to 10 and AN thereafter, the feeding protocol used in the present study, is a more simple method to rare first-feeding zebrafish larvae compared to current published methods especially if zebrafish is maintained in low-cost facilities.

LAN looks a suitable alternative for zebrafish larviculture although there are some negative points to recruit that for zebrafish low-cost facilities: (1) it is expensive,[80] (2) strongly contaminates larval culture medium, a problem that is cited for processed feeds as well[6,22] that leads to a complete larvae medium exchange after each feeding, a laborious and time consuming work, (3) it can be a nutritional problem in view of its deficiency in sulphur amino acids, like methionine[81] although this can be addressed by combining the LAN with lyophilized adult *Artemia* to feed first-feeding larvae, and (4) the preparation of LAN needs a freeze drying system that is not always available in a low-cost facility.

Egg yolk per se is highly nutritious and is unquestionably one of the most nutritionally balanced foods known for man and animals. The fine granular texture of boiled yolk have indeed provided aquaculturists with a practical artificial diet that is superior to most other artificial feeds for this purpose.[80] In addition, the similar survival and growth rates of YOLK- and LAN-fed larvae, together with lacking the above mentioned shortcomings of LAN, uncovers the potential of YOLK as a starter food for zebrafish first-feeding larvae in low-cost facilities.

As a conclusion, the results of the present study show that ALG, either alone or in combination to LAN (ALG+LAN group), cannot be considered as a suitable starter food for zebrafish. YOLK might be recommended as the best one.

Acknowledgments

The authors are grateful to Dr. Fatemeh Mansouri (Urmia University of Medical Sciences, Iran) for her help to design primers.

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TABLES

Table 1. List of primer sets used in this study for real-time PCR.

Footnote → **F**: Forward; **R**: Reverse primer.

Table 2. The value of physico-chemical parameters of culture medium before and one hour after adding food groups to culture container of larvae.

Footnote → Data are mean ± SD. Mean values within horizontal rows superscripted by the same letter are not significantly different. YOLK: egg yolk; LAN: lyophilized "*Artemia* nauplii; AALG: alive algae; LALG: lyophilized algae; AALG+LAN: a combination of AALG and LAN; LALG+LAN: a combination of LALG and LAN.

Table 3. Fatty acid (FA) composition of four freshwater algae used to prepare green water and *Artemia* nauplii for zebrafish larviculture.

Footnote → Data are mean ± SD. Mean values within horizontal rows superscripted by the same letter are not significantly different. (n): Number of specimens for each alga and AN; *CV*: *Chlorella vulgaris*; *SO*: *Scenedesmus obliquus*; *HP*: *Haematococcus pluvialis*; *SP*: *Spirulina sp*.

Table 4. Hatching rate (HR, Mean±SD) of zebrafish larvae cultured using system water and microalgae (green water technique) at different days post fertilization.

Footnote → Different superscript letters indicate significant differences. See section 2.3 for the methods used in zebrafish larviculture.

Table 5. Survival rate (SR%, days 7 to 11 post fertilization [dpf]) and morphometric characteristics (MCs, 11dpf) of zebrafish larvae fed on six algal groups: three alive algae (ACV, ASO, and AHP) and three lyophilized algae (LCV, LSO, and LHP)..

Footnote → Mean±SD, different superscript letters indicate significant differences. Number of specimens (n) for SR and MCs are 200 and 6, respectively. alive microalgae (alive *C. vulgaris* [ACV], *S. obliquus* [ASO], and *H. pluvialis* [AHP]), lyophilized microalgae (lyophilized *C. vulgaris* [LCV], *S. obliquus* [LSO], and *H. pluvialis* [LHP]). See the legend of the figure 1 for abbreviations of the MCs.

Table 6. Survival rate (SR%, days 7 to 11 post-fertilization [dpf]) and morphometric characteristics (MCc, 11 dpf) of zebrafish larvae fed on six algal groups combined with LAN: three alive algae combined with LAN (ACV+LAN, ASO+LAN, and AHP+LAN) and three lyophylised algae combined with LAN (LCV+LAN, LSO+LAN, and LHP+LAN).

Footnote → Mean ± SD, different superscript letters indicate significant differences. Number of specimens (n) for SR and MCs are 200 and 6, respectively. LAN: lyophilized *Artemia* nauplii. A combination of alive microalgae (alive *C. vulgaris* [ACV], *S. obliquus* [ASO], and *H. pluvialis* [AHP]) and LAN (i.e. ACV+LAN, ASO+LAN, and AHP+LAN), and a combination of lyophilized microalgae (lyophilized *C. vulgaris* [LCV], *S. obliquus* [LSO], and *H. pluvialis* [LHP]) and LAN (i.e. LCV+LAN, LSO+LAN, and LHP+LAN). See the legend of the figure 1 for abbreviations of the MCs.

Table 7. Comparison among zebrafish larvae fed on different food groups concerning morphometric characteristics (MCs) on day 11.

<u>Footnote</u> → Data are mean ± SD. Mean values within horizontal rows superscripted by the same letter are not significantly different. Number of specimens (n) for MCs are 6. YOLK: egg yolk, LAN: lyophilized *Artemia* nauplii, ALG: alga,; STA: starved. BL (total body length): largest horizontal body distance — anterior part of the head to the end of the body. APB (anterior part of the body): anterior part of the head to the posterior insertion of yolk sac. PoPB (posterior part of body): the posterior insertion of yolk sac to the end of the body. HL (head length): anterior part of the head to the place where the head is connected to the body. BD1 (body depth 1): vertical distance from posterior insertion of yolk sac to the upper surface of the body. BD2 (body depth 2): largest vertical body distance. Fifteen ratios were calculated from the six morphometric characteristics and included in Tables 6 and 7 BL/APB, BL/PoPB, BL/BD1, BL/BD2, BL/HL, APB/PoPB, APB/BD1, APB/BD1, APB/HL, PoPB/BD1, PoPB/BD2, PoPB/HL, BD1/BD2, BD1/HL, and BD1/HL.

FIGURES

Fig. 1. Larval feeding period in the current study and four different methods of used to feed larvae with alive algae.

Fig. 2. Morphometric characteristics determined in zebrafish embryos and larvae. The landmarks drawn on the schematic of zebrafish larvae at 120 hpf depict the characteristics that were utilized for screening IM-induced responses. BL (total body length): largest horizontal body distance — anterior part of the head to the end of the body. APB (anterior part of the body): anterior part of the head to the posterior insertion of yolk sac. PoPB (posterior part of body): the posterior insertion of yolk sac to the end of the body. HL (head length): anterior part of the head to the place where the head is connected to the body. BD1 (body depth 1): vertical distance from posterior insertion of yolk sac to the upper surface of the body. BD2 (body depth 2): largest vertical body distance. Fifteen ratios were calculated from the six morphometric characteristics and included in Tables 6 and 7 BL/APB, BL/PoPB, BL/BD1, BL/BD2, BL/HL, APB/PoPB, APB/BD1, APB/BD1, APB/HL, PoPB/BD1, PoPB/BD2, PoPB/HL, BD1/BD2, BD1/HL, and BD1/HL.

Fig. 3. Results in survival rate (SR) of zebrafish larvae at different days post fertilization. YOLK: egg yolk, LAN: lyophilized *Artemia* nauplii, ALG: algae, STA: starved. Different letters indicate significant differences among the groups (ANOVA; $P<0.05$).

Fig. 4. Photomicrographs of zebrafish larvae at 8 (A), 9 (B), and 11 (C) days post fertilization (dpf). The arrows in A indicate ingested algae by a 8 dpf zebrafish larva. B algae fed larvae at 9 dpf releasing fecal droppings. C algae fed larvae at 11 dpf.

Fig. 5. Digestive enzyme activities in zebrafish larvae. YOLK: egg yolk, LAN: lyophilized *Artemia* nauplii, ALG: algae, STA: starved, BF: before feeding. Results that do not share the same letter are significantly different.

Fig. 6. Variation among food groups concerning morphometric characteristics (MCs) at 11 dpf (days post-fertilization) (A, C, E, G, I). Results that do not share the same letter are significantly different. Scatter plots show significant relationships between survival rate (SR) at 11 dpf (as dependent variables) and MCs at 11 dpf (as the independent variable) (B, D, F, H, J).YOLK: egg yolk, LAN: lyophilized *Artemia* nauplii, ALG: algae, STA: starved. See the legend of the figure 1 for abbreviations of the MCs.

Fig. 7. Variation among food groups concerning the expression level of *vegfaa* (A) and *igf2a* (C) at 11 dpf (days post-fertilization). Results that do not share the same letter are significantly different. Scatter plot shows significant relationships between survival rate (SR) at 11 dpf (as dependent variables) and *vegfaa* at 11 dpf (as the independent variable) (B). Scatter plots show significant relationships between morphometric characteristics (MCs) at 11 dpf (as dependent variables) and *igf2a* at 11 dpf (as the independent variable) (D-G). YOLK: egg yolk, LAN:

lyophilized *Artemia* nauplii, ALG: algae, STA: starved. See the legend of the figure 1 for abbreviations of the MCs.