Early transition to microdiets improves growth, reproductive performance and reduces skeletal anomalies in zebrafish (*Danio rerio*)

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Abstract

Zebrafish is a model species with a high variability of feeding regimes among fish facilities. The use of live feeds for early life stages is a common practice and few studies have focused early weaning into microdiets. The lack of standardized feeding protocols amongst research facilities promotes discrepancies in biological performances and few studies relate dietary regimes to zebrafish development. The objective of this work was to assess the effect of an early transition into microdiets in zebrafish development by evaluating growth, survival, reproductive performance and skeletal anomalies. These parameters were assessed in one group exclusively fed on *Artemia* nauplii and two groups fed on microdiets (commercial and experimental). Results showed that an early weaning with the two microdiets significantly improved zebrafish

growth and reproductive performance, while a decrease in incidence of vertebral column anomalies was observed. A high survival was also maintained in fish fed microdiets at an early developmental stage when comparing to exclusive *Artemia* nauplii feeding. In conclusion, early weaning with high quality microdiets is beneficial for zebrafish growth, reproductive performance and skeletal development, contributing to the standardization of zebrafish husbandry practices.

Keywords: microdiets, skeletal anomalies, reproduction, growth, zebrafish feeding protocol

Introduction

Zebrafish is an important model species in numerous areas, including developmental biology, ecotoxicology, neurobiology, biomedicine and aquaculture.[1–5] In the past two decades, the development of novel technologies and molecular tools contributed to the increase in relevance of this species in biomedical research.[1–4] However, procedures concerning zebrafish husbandry, such as feeding protocols and nutritional composition of diets utilized, vary markedly among fish facilities.[1,6–10] This lack of standardization in husbandry procedures leads to a high degree of variability in fish growth performance and reproductive success, and until today the modulation of zebrafish dietary requirements is still poorly addressed.[10,11] The broodstock diet is highly relevant in teleosts, not only to its health state but also to the quality of its gametes and progeny.[12–14] The maintenance of body homeostasis is affected by the interaction between nutrition, metabolism, gene expression and epigenetic changes that modulate intracellular signaling pathways.[14] In this sense, it has been proposed a possible biological mechanism of nutritional “imprinting” events that modulate gene expression and

epigenetic patterns that could be transmitted to the progeny.[15–18] Therefore, nutrition research is highly relevant towards the standardization of zebrafish rearing, which can be achieved through the use of microdiets with controlled nutritional composition.

Zebrafish larvae are commonly fed with live preys including paramecia (*Paramecium sp.*), rotifers (*Brachionus sp*.) and *Artemia* nauplii (*Artemia sp*.)[1,6,8,11,19–21] until weaning at subadult stage (~30 days post-fertilization).[22] After weaning, juvenile and adult feeding may rely on a wide variety of diets, from flakes for aquarium species, to extruded microdiets, often primarily developed for aquaculture species.[3,23] When both diet types are compared, extruded diets generally result in improved larval quality and growth performance, as well as a superior water quality.[9] Moreover, a continuous supply of live feeds is often common in zebrafish feeding during the juvenile and adult stages, namely concerning *Artemia* nauplii. This strategy contributes as an environmental enrichment factor, stimulating the natural predatory behavior of fish[10,24,25] and lowering stress related to captivity, thus improving fish welfare.[26] These different feeding protocols implemented in fish facilities resulted in different nutritional compositions that may affect development. The use of a standardized diet in zebrafish rearing facilities is of utmost importance to increase the reproducibility of research conducted with this model.[11]

High reproductive performance in zebrafish is one of the most desired outcomes amongst the research community, since embryos are often the main focus of developmental studies, also frequently being a limiting factor in experimental designs.[11] For this purpose, the ultimate goal for zebrafish is to reach the adult stage in a short period of time, or to modulate and enhance its reproductive performance through the dietary regime.[22] Diet composition provided to zebrafish breeders is extremely important for egg production, fertilization and hatching rates.[13,27–29] For instance, the

presence of specific phospholipids in the diet were shown to be essential for improving zebrafish sperm quality and reproductive performance.[13] On the other hand, a diet based on flakes led to a negative effect in zebrafish reproduction by reducing egg production.[20] Furthermore, the inclusion of *Artemia* nauplii in the dietary regime lead to an improvement of gamete production, fertilization rates and spawning performance in zebrafish.[23,27] The continuous improvement of microdiets is essential to increase the zebrafish reproductive performance.[3,11,25]

Zebrafish has also been successfully used as a model to understand cellular and genetic aspects of vertebrate skeletogenesis,[30,31] since it has a mineralized bone matrix similar to mammals, with both endochondral and intramembranous ossification as well as functional osteoblasts, osteocytes and osteoclasts.[5,31–33] However, little is known about the effect of the dietary regime on zebrafish skeletal development.[13,34] Therefore, this work aimed at evaluating the effect of an early transition from live feeds (*Artemia* nauplii) to microdiets (commercial and experimental) and their impact on skeletal formation in zebrafish larvae when compared to a feeding regime exclusively based on *Artemia* nauplii. In addition, this study evaluated the effect of these dietary treatments on zebrafish growth and reproductive performance.

Material and Methods

Ethics Statement

All animal manipulations were performed in compliance with the Guidelines of the European Union Council (86/609/EU) and transposed to the Portuguese law for the use of laboratory animals on research by "Decreto Lei n° 129/92 de 06 de Julho, Portaria n° 1005/92 de 23 de Outubro", and according to the European parliament council directive´s for protection of animals used for scientific research (2010/63/EU). All

101 animal protocols were performed under a "Coordinator-researcher" license from the
102 Direção-Geral de Veterinária, Ministério da Agricultura, do Desenvolvimento Rural e
103 das Pescas, Lisbon, Portugal, under the "Decreto Lei n°113/2013 de 7 de Agosto"
104 relative to the protection of animals used for scientific research.
105
106 *Housing conditions*
107 A breeding population of zebrafish wild-type AB strain (ZFIN ID: ZDB-GENO-
108 960809-7) maintained at the Centre of Marine Sciences (CCMAR, Portugal) for more
109 than 10 generations was used to generate the embryos used in the trial. The fish room
110 had a controlled photoperiod with a 14:10 hour light:dark cycle and humidity close to
111 60%.[35] Fish were housed in 3.5 L tanks placed in a 980 L recirculating system
112 (ZebTEC®, Tecniplast, Italy). The water quality was maintained by partial water
113 renewal (10% of total volume daily) and through filtration: biological filtration (ceramic
114 beads), mechanical filter (pleated cartridge filters, 50 μm), carbon filter (granular
115 activated carbon filter) and ultraviolet sterilization (180 000 μWs/cm^2). Water
116 conditions were as follows: temperature: 28.0 ± 1 ºC; pH 7.5 ± 0.2; and conductivity
117 750 ± 30 μS. Nitrogen compounds were monitored weekly, presenting values constantly
118 below 0.1 mg/L (NO_2^- and NH_4^+) and below 50 mg/L (NO_3^-) throughout the
119 experimental period.
120
121 *Fish rearing and diet preparation*
122 A broodstock group of AB strain males (n = 15) and females (n = 15), between 4 - 5
123 months, were crossed and approximately 1000 eggs were collected and incubated at
124 28.0

± 0.5 ºC in 1L nursery tanks (density of 200 eggs/L) with E2 embryo medium containing 50 ppt of methylene blue (Sigma-Aldrich, Spain) to reduce bacterial and fungal growth.[13,19] At 5 days post-fertilization (dpf), 900 larvae were pooled and divided into triplicates (100 larvae/L) for each treatment group.

The experimental design comprised 3 different treatment groups: the first group, was fed with 3 meals of *Artemia* nauplii per tank (AF 480; INVE, Belgium) with a supply of 5000 nauplii per meal between 5 and 10 dpf. Between 11 and 20 dpf, 10 000 nauplii per meal were supplied; between 21 and 30 dpf, 15 000 nauplii were supplied per meal. From 30 dpf until the end of the breeding trials, the fish were fed two meals containing 40 000 *Artemia* nauplii per tank (Fig. 1). Commercial diet (CD) and Experimental diet (ED) groups were reared in a co-feeding regime (5 and 8 dpf) with 5000 *Artemia* nauplii per tank once a day, and twice a day with extruded diets. From 8 to 30 dpf fish were fed with 3 meals a day with microdiets representing 15 to 20% of larvae body weight. From 30 dpf until the end of the breeding trials each fish tank was fed with microdiets representing 3 to 5% of fish body weight (Table 1).

The CD contained the following ingredients: fish meal, lecithin, wheat gluten, dried seaweed, fish oil, maize starch, vitamins and minerals. The ED was produced using the following main ingredients: fish meal, fish solubles, lecithin, wheat gluten, vitamins and mineral premixes. Briefly, the ED was produced by Sparos Lda (Olhão, Portugal) using extrusion at low temperatures as main production process. Powder ingredients were mixed in a double helix mixer and ground in a micropulverizer hammer mill (SH1, Hosokawa-Alpine, Germany). The powders were humidified and agglomerated by low temperature extrusion (Dominioni Group, Italy). Resultant pellets were dried in a convection oven (OP 750-UF, LTE Scientifics, United Kingdom) for 4 h at 60 ºC, crumbled (Neuero Farm, Germany) and sieved to desired size ranges (<100 μm, 100 –

150 200 µm, 200 – 400 µm and 400 - 600 µm). These size ranges were adapted according to
151 fish mouth size and developmental stage. Proximate composition of dietary treatments
152 is shown in Table 2.
153
154 *Reproduction trials*
155 At 120 dpf, fish were divided according to their sex, based on to the differences in
156 morphology and pigmentation.[22] The reproductive performance trials started when fish
157 were prone for mating events (3 - 4 month old). Males and females were housed in
158 separated 3.5 L tanks to improve their reproductive efficiency.[36] Two breeding groups
159 of 2 males and 3 females were randomly chosen from population and set up in a
160 standard 1 L breeding tanks (Tecniplast, Italy) (n = 5 crosses) 15 h before the spawning
161 period (adapted from Lawrence *et al*.[22]). Couples were allowed to mate 1 h after the
162 beginning of the light phase by removing the plastic partition that kept both sexes
163 separated. Fish returned to their respective housing tanks 2 h after the beginning of the
164 spawning period, being crossed with 15 days of interval between spawning events to
165 maximize gametes release. The eggs were collected and incubated as previously
166 described. At 3 dpf, the number of hatched embryos was determined under a
167 stereomicroscope (Leica MZ6, Leica, Germany).
168 Zebrafish larvae were raised in static conditions in 1 L breeding tanks between 5 to 15
169 days post-fertilization (100 larvae/L) with daily water renewal (50%). At 15 dpf, larvae
170 were transferred to 3.5 L tanks (25 larvae/L) in a ZebTEC recirculating system with a
171 flow rate of 150 mL/min until fish reached the adult stage (3-4 months).
172
173 *Fish sampling*

A group of 10 larvae from each tank (n = 30 observational units) were sampled for standard length (SL) at 15, 30, 60 and 120 dpf. Larvae were photographed using a Digital camera (Canon Power shot G12, Canon, Japan) attached to a stereomicroscope (Leica MZ6, Leica, Germany), and images were analyzed using ZEISS AxioVision (version 4.8, Carl Zeiss, Germany). The groups of larvae with 15 and 30 dpf were euthanized with a lethal dose of tricaine methanesulfonate (MS-222; Sigma-Aldrich, Spain) and stored at -20 °C, freeze-dried, and weighted to determine dry weight (DW). Juvenile (60 dpf) and adult fish (120 dpf) were anesthetized with 150 mg/L of MS-222, measured and weighted.

To evaluate larvae skeletal anomalies, 30 larvae per tank (n = 90 observational units) were sampled at 30 dpf, euthanized with a lethal dose of MS-222 and fixed in a 4% buffered paraformaldehyde solution at 4 °C for 24 h. Larvae were subsequently washed with a phosphate buffer saline 0.1 M, pH 7.4 solution and stored in 75% ethanol at room temperature (adapted from Gavaia *et al*.[37]). Whole-mount acid-free double staining was performed using alcian blue 8GX (Sigma-Aldrich, Spain) for cartilage and alizarin red S (Sigma-Aldrich, Spain) for mineralized bone.[38] Briefly, samples were stained in alcian blue 8GX for 1.5 h and passed through a decreasing series of ethanol concentrations (96 to 25%), and hydrated with distilled water before being stained overnight with alizarin red S in a potassium hydroxide solution (KOH) (Sigma-Aldrich, Spain) at 0.5%. Samples were cleared with a 0.5% KOH solution and stored in a solution of 90% glycerol (Merk Millipore, Billerica, MA) at room temperature. The detection of skeleton anomalies was performed following the nomenclature by Bird and Mabee[39] and Bensimon-Brito *et al*.[40].

Data analysis

Results were expressed as means ± standard deviation (SD). Data normality was tested using a Kolmogorov-Smirnov test. Mean differences between treatments for fish growth and length were analyzed using a Kruskal-Wallis test followed by a Mann-Whitney U Test ($p < 0.05$). Statistical differences between treatments for skeletal anomalies were evaluated with Person´s Chi-squared test ($p < 0.05$). Significant differences between treatments for the number of eggs and the number of eggs per female was evaluated using a Student´s *t*-test ($p < 0.05$). IBM SPSS Statistics 25.0 software was used for data and statistical analysis.

Results

Larval performance

No significant differences were observed between treatments for fish survival during the course of the experiment (Table 3). At 15 and 30 dpf, fish fed CD had a significantly higher standard length (SL) than larvae fed with *Artemia* nauplii and ED (Table 3). No significant differences were observed for SL of fish from the ED and Artemia treatment at 15 DPF. However, at 30 dpf, the ED dietary treatment resulted in higher SL values than in the *Artemia* nauplii treatment (Table 3). No significant differences were observed between CD and ED treatments until the end of the experiment (60 and 120 dpf), in which both treatments obtained higher SL values than larvae fed with *Artemia* nauplii (Table 3). There were no significant differences between treatment groups regarding dry weight at 15, 30 and 60 dpf (Table 3). Significant differences were observed when fish reached 120 dpf, where fish fed with CD and ED showed a significantly higher weight than fish fed with *Artemia* nauplii (Table 3).

222 At 120 dpf, no statistical differences were observed regarding fish sex ratios of the
223 progeny obtained by breeders from the different dietary treatments. However, a higher
224 number of males was observed in all treatments (Table 3).
225
226 *Reproductive performance*
227 Fish fed with CD and ED presented a significantly higher number of spawned eggs, as
228 well as a higher female contribution, when compared to fish fed with the *Artemia*
229 nauplii feeding regime (Table 4). However, there were no statistical differences in
230 embryo hatching rate observed between the different treatments (Table 4).
231
232 *Skeletal development*
233 Skeletal evaluation was performed at 30 dpf, when all skeletal structures were
234 completely formed. Fish fed with *Artemia* nauplii showed a significantly higher
235 incidence of total skeletal anomalies (90.00 ± 10.00%) than fish fed with CD (48.33 ±
236 1.67%) and ED (51.11 ± 4.44%) feeding regimes (Fig. 1 A). No statistical differences
237 were observed for skeletal anomalies between fish fed with both microdiets (Fig. 1 A).
238 There were no significant differences observed in the distribution of skeletal anomalies
239 throughout the zebrafish vertebral column between treatment groups. However, most of
240 the detected anomalies were found in the fish posterior region (caudal fin vertebrae)
241 (Fig. 1 B). No statistical differences were observed in the number of anomalies between
242 the different treatment groups (Fig. 1 C). The most common anomalies observed were
243 vertebral fusions, compressions, lordosis, scoliosis and shortened vertebrae. Vertebral
244 fusions were identified by the presence of secondary neural arches. Compressions
245 caused deviations to the normal pattern of the vertebral column (Fig. 2).
246

Discussion

This work assessed the viability of an early introduction of microdiets and live feed replacement in the dietary regime of zebrafish, a widely used model in biomedical research. Although live feeds such as paramecia, rotifers or *Artemia* nauplii are a common practice in zebrafish husbandry. Results from the current study showed benefic effects of using a short co-feeding regime with microdiets and *Artemia* nauplii followed by an early transition to a feeding regime composed solely by microdiets (CD and ED). An early transition to microdiets increased larvae growth, maintaining a high survival and lower prevalence of skeletal anomalies when compared to exclusive live feed (*Artemia* nauplii) regime. Therefore, shortening the live feed administration period is beneficial in zebrafish larvae rearing.

The transition from live feeds to microdiets is known to be a sensitive period in fish development. Few studies were conducted with an earlier transition from live feeds to commercial or experimental extruded diets, especially at the onset of exogenous feeding in zebrafish larvae.[10,23,41] In our study, the highest standard length values at 15 dpf were achieved by larvae fed with CD (6.34 ± 0.45 mm) when compared with larvae fed the ED (5.89 ± 0.53 mm) and *Artemia* nauplii (6.11 ± 0.68 mm). Despite the differences in rearing densities, the observed values of larvae length in the CD treatment group were comparable to those obtained by Kaushik *et al*.[23] (approximately 8 mm) at the same age (15 dpf) with a similar feeding protocol. Moreover, results from the current study are also similar to those obtained by Gómez-Requeni *et al*.[41], who used a commercial diet (JBL Novo Tom Artemia diet; JBL GmbH & Co., Germany) until 16 dpf, achieving a fork length of 6.84 mm. The larval growth observed in our study, was identical to Kaushik *et al*.[23] at 60 dpf (approximately 22.5 mm). Consequently, the dietary protocol

proposed in the current study reflects a normal zebrafish larval growth when compared to previously conducted studies.

Broodstock nutrition is an essential factor to optimize breeder´s reproductive ability, improving thus gamete quality and fertilization rates, as well as the progeny quality.[12,13] More specifically, the diet composition is known to affect reproductive performance in zebrafish in terms of clutch size, hatching rate and consequently larval growth performance.[10,13,29] In our feeding trial we observed that both microdiets (CD and ED) achieved significantly higher number of eggs spawned when compared to fish fed exclusively on *Artemia* nauplii, yielding a clutch size above zebrafish average (approximately 200 eggs/female).[36] The lower number of eggs observed in *Artemia* nauplii treatment may be related to its suboptimal nutritional composition,[27–29] which is known to have impact on fish reproduction.[20] This suggest that *Artemia* nauplii does not fulfil the nutritional requirements necessary for optimal oocytes production and quality. It is known that highly unsaturated fatty acids (HUFAs) and phospholipids have a particularly relevant role in zebrafish broodstock nutrition, since they improve reproduction performance and gametes quality.[13,29,42] Arachidonic acid (ARA), eicosapentaenoic acid (EPA) and docosahexaenoic acid (DHA) are involved in reproduction processes such as oocyte maturation, ovulation, spawning, hatching success and larval quality.[43,44] Zebrafish have the ability to biosynthesize EPA and DHA from α-linolenic acid (LNA, 18:3n-3) and ARA from linoleic acid (LA, 18:2n-6). The biosynthesis extent is dependent on the activities of desaturase and elongase enzymes, however the rate at which this biosynthesis occurs remains to be established.[45,46] Ishak *et al*.[46] observed higher ARA level in pre-vitellogenic and matured follicles while DHA level were higher during late vitellogenic and maturation stage, consequently HUFA synthesis is involved in oocyte maturation and ovulation.

296 Since *Artemia* nauplii contain low DHA and higher ARA composition,[47] we suggest
297 that the low content in DHA might compromise oocyte late maturation. Furthermore,
298 the inclusion of those HUFA´s in zebrafish diet may contribute to a reduction of
299 metabolic effort invested in biosynthesis and favoring the metabolic investment on
300 gamete production. It is known that dietary phosphatidilcholine and
301 phosphatidilethanolamine supplementation improve significantly reproductive
302 performance and sperm quality in zebrafish.[13] Therefore, not only the diet total lipid
303 content but also the specific lipid categories and their ratios might play an important
304 role on zebrafish reproduction.[48] The fact that the nutritional composition of microdiets
305 is easier to modulate than the nutritional profile of live feed, indicates that microdiets
306 can be an important nutritional tool to improve the reproduction of zebrafish.
307 During zebrafish development, the timing of ingestion of specific nutrients as well as
308 the bioavailability of certain nutrients (e.g. lipids, amino acids, vitamins and minerals)[49]
309 may affect the process of skeletal formation. The majority of the skeletal anomalies
310 found in this study were located in caudal fin vertebrae, with the presence of fusions in
311 the last vertebrae, scoliosis and deviations in relation to other vertebrae. The remaining
312 affected structures presented lordosis and vertebral compressions caused by
313 compression forces with consequent abnormal vertebra formation. Zebrafish is
314 particularly susceptible to the incidence of skeletal anomalies in caudal fin vertebrae,
315 has previous reported.[50] Despite the predominant number of anomalies in the pre-caudal
316 fin vertebrae, zebrafish fed exclusively with live feed (*Artemia* nauplii) showed a
317 significantly higher prevalence in skeletal anomalies when compared to fish fed with
318 CD and ED. *Artemia* nauplii is known to lack essential nutrients, such as selenium, zinc,
319 copper and manganese that are important for fish development and skeletogenesis.[51]
320 Moreover, as previously mentioned, *Artemia* nauplii composition in HUFAs may be

unsuitable for a correct bone formation. Since high EPA levels inhibit the extracellular matrix mineralization and a high DHA content is required for a correct bone formation by altering the cell phenotype, gene expression and mineralization capacity. Inadequate levels of these HUFAs in *Artemia* nauplii are likely related to an incorrect skeletogenesis.[49,52] The same relationship between DHA and the correct bone formations were observed by Izquierdo *et al*.[53] with a decrease in 50% the number of skeletal anomalies of red porgy with higher DHA supplementation in the diet. However, the EPA and DHA requirements differ between marine and freshwater species and comparisons on the effects of these dietary factors in marine/freshwater fish development should be taken carefully. Still, comparing the nutritional profile of *Artemia* nauplii and microdiets used in the current study, it is possible to observe that mineral content in *Artemia* nauplii represents only approximately 35-40% of the values observed in the microdiets. Future studies should understand if these reduced levels in the total mineral content or in specific minerals such as calcium, phosphorous or respective ratio; are responsible for the higher prevalence of skeletal anomalies in zebrafish fed with *Artemia* nauplii. Nevertheless, like in HUFAs, it is possible that the mineral fraction of Artemia nauplii may be inadequate for a correct skeletal development of zebrafish and its use as main dietary source should be avoided in zebrafish husbandry, especially in studies assessing skeletal development. Further research is required to establish the nutritional requirements of zebrafish, thus allowing to improve microdiets used in zebrafish husbandry, contributing for an adequate development and skeletogenesis. The standardization of zebrafish nutrition is a pressing matter, live feeds are labor intensive and prone to pathogenic contaminations which can compromise fish health.[25] Since microdiets are practical, nutritionally controlled and expected to present lower biosecurity risks, they are a promising tool for standardization

purposes. Ultimately, the development of standardized high quality microdiets specific for zebrafish would lead to a higher experimental reproducibility in rearing methodologies between research facilities.

In conclusion, this study showed that an early transition to microdiets significantly improved zebrafish growth and reproductive performance, while decreasing the number of vertebral column anomalies and maintaining a high survival when compared to the *Artemia* nauplii feeding regime. This study therefore, contributes to the improvement of zebrafish husbandry by ameliorating zebrafish development, quality and reproduction through an early introduction of microdiets.

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Disclosure Statement

No competing financial interests exist

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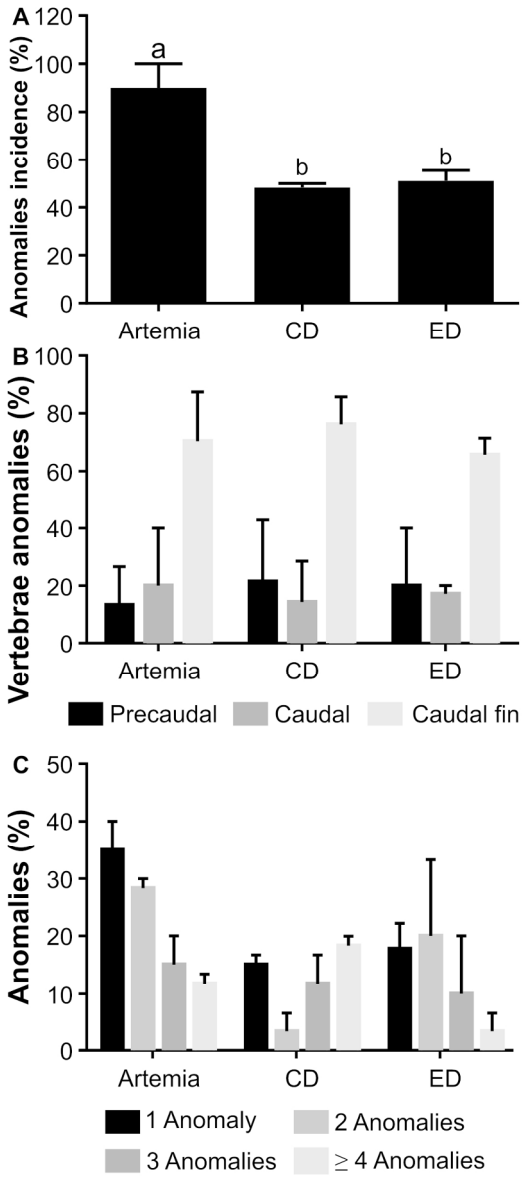

FIG 1 - Zebrafish skeletal anomalies detected at 30 dpf in Artemia nauplii (Artemia), Commercial Diet (CD) and experimental diet (ED) treatments in terms of: (A) Total incidence of anomalies (%), (B) Anomalies distribution in the vertebral column (%), (C) Load of anomalies in the vertebral column (%). Statistical differences between treatments in the total incidence of anomalies was evaluated with Pearson´s chi-squared test ($p < 0.05$).

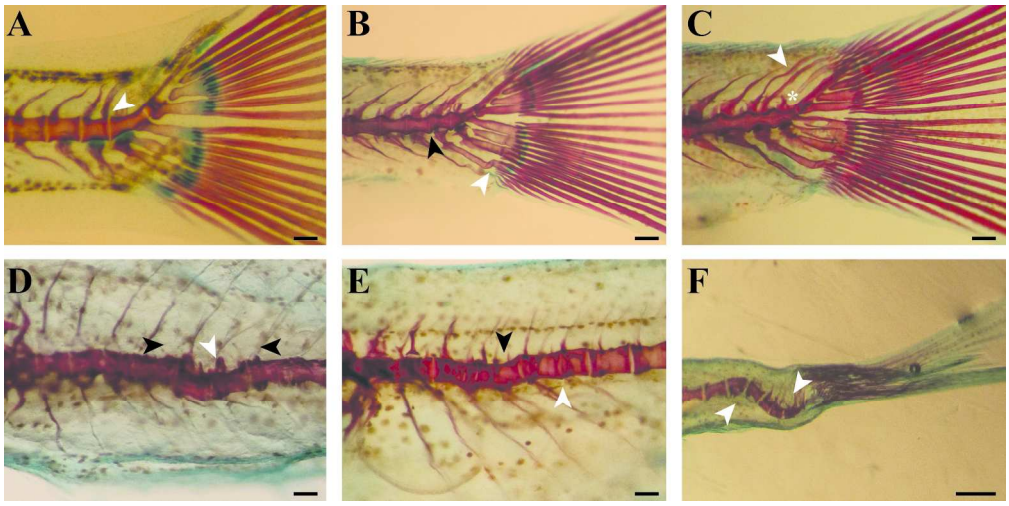

FIG 2 - Zebrafish most common skeletal anomalies observed at 30 dpf in Artemia nauplii (Artemia), Commercial Diet (CD) and experimental diet (ED), detected by the double staining (Alcian blue and Alizarin red S). Cartilage is stained in blue and mineralized bone is stained in red. (A) Fusion in the last caudal fin vertebra, No. 29 -30, identified by the presence of secondary neural arches (white arrow, CD) (B) Caudal fin vertebral deviation in relation to other vertebra, No. 30 (compression; black arrow); severe anomaly of the haemal arche, that supportsthe caudal fin (white arrow; Artemia) (C) Secondary neural arche in the last caudal fin vertebrae, No. 30 (white arrow); existence of a broken neural arche (asterisk; Artemia) (D) Lordosis in precaudal vertebrae, No. 7 and 8 (white arrow), associated to a vertebral compression (black arrows; ED) (E) Precaudal vertebral compression, that led to an abnormal vertebra formation, No. 8 (black arrow); presence of a short length vertebrae, No. 9 (compression) (white arrow; CD) (F) Scoliosis in caudal fin vertebrae (compression, white arrows; CD). Scale bars = 0.1 mm

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Table 1 - Zebrafish experimental feeding protocol

Treatment	*Artemia nauplii*		*CD*		*ED*	
Age (dpf)	Diet	Meals	Diet	Meals	Diet (μm) and artemia	Meals
5 - 10	5000 art/tank	3x	75 μm	2x (25 mg)	< 100 μm	2x (25 mg)
			5000 art/tank	1x	5000 art/tank	1x
10 – 20	10 000 art/tank	3x	150 μm	3x (75 mg)	100 – 200 μm	3x (75 mg)
20 - 30	15 000 art/tank	3x			200 – 400 μm	3x (75 mg)
30 - 45	40 000 art/tank	2x				
45 - 120			300 μm	3x (175 mg)	400 – 600 μm	3x (175 mg)

Artemia, *Artemia* nauplii AF480 (n = 3); CD, Commercial diet (n = 3); ED, Experimental diet (n = 3)

Table 2 - Proximate compositions of dietary treatments

Treatment	*Artemia* nauplii	*CD*	*ED*
Crude protein (g/100 g, DM)	54	59	63
Total lipid (g/100 g, DM)	12	14	20
Ash (g/100 g, DM)	5	14	14
Crude fiber (g/100 g, DM)	-	0.2	0.1
Gross energy (KJ/100 g, DM)	-	20.8	21
ARA (20:4 n-6, g/100 g, DM)	0.9 – 1.3	0.1	0.05
EPA (20:5 n-3, g/100 g, DM)	0.3 – 2.4	1	0.5
DHA (22:& n-3, g/100 g, DM)	0.4	2.3	0.8

Artemia, *Artemia* nauplii AF480; CD, Commercial diet; ED, Experimental diet; DM, dry matter; ARA, Arachidonic acid; EPA, Eicosapentaenoic acid; DHA, Docosahexaenoic acid

*Camargo *et al*.[55]

Table 3 - Growth, weight and survival performance

Treatment	*Artemia*	*CD*	*ED*
15 dpf			
Mean SL (mm)	6.11±0.68[b]	6.34±0.45[a]	5.89±0.53[b]
Mean DW (mg/larvae)	0.31±0.00	0.19±0.03	0.20±0.02
Survival (%)	77.33±2.87	80.33±2.62	77.67±2.87
30 dpf			
Mean SL (mm)	6.38±1.10[c]	8.94±1.34[a]	8.03±1.14[b]
Mean DW (mg/larvae)	1.28±1.17	1.93±1.70	1.96±1.81
Survival (%)	76.33±2.49	79.00±1.63	76.67±2.62
60 dpf			
Mean SL (mm)	14.34±2.61[b]	23.41±2.62[a]	21.76±2.21[a]
Mean DW (mg/larvae)	52.29±27.53	78.93±51.36	86.74±46.03
Survival (%)	76.33±2.49	79.00±1.63	76.67±2.62
120 dpf			
Mean SL (mm)	26.67±1.76[b]	33.00±1.47[a]	31.26±6.06[a]
Mean DW (mg/larvae)	344.00±147.78[b]	600.79±136.20[a]	498.57±164.25[a]
Survival (%)	76.33±2.49	79.00±1.63	76.67±2.62
Sex-ratio (% of males)	25.00±6.25	28.99±0.42	37.09±1.37

Data are mean±SD

Statistical differences (Kruskal-Wallis test followed by a Mann-Whitney U Test, $p < 0.05$) are represented by letters

dpf, days postfertilization; SL, standard length; DW, dry weight; *Artemia*, *Artemia* nauplii (n = 3); CD, Commercial diet (n = 3); ED, Experimental diet (n = 3)

Table 4 - Zebrafish reproductive performance at 120 days post-fertilization

Treatment	*Artemia*	*CD*	*ED*
No. of reproductive events	5/5	5/5	5/5
No. eggs spawned	128.80±47.27[b]	257.40±79.80[a]	353.80±94.13[a]
Mean female contribution	68.00±16.63[b]	128.70±39.90[a]	131.45±22.30[a]
Hatching rate (%)	82.60±11.06	90.20±5.91	93.20±5.42

Data are mean±SD

Statistical differences (Student´s *t*-test, $p < 0.05$) are represented by letters

Mean female contribution, total number of eggs/number of females

Artemia, *Artemia* nauplii; CD, Commercial diet; ED, Experimental diet