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Balancing between *Artemia* and microdiet usage for normal skeletal development in zebrafish (*Danio rerio*)

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Abstract :

Targeting in zebrafish fast growth, high survival rates and improved reproductive performance has led over the last years in variable feeding regimes between different facilities. Despite its significance on fish function and welfare, normal skeletal development has rarely been evaluated in establishing the best feeding practices for zebrafish. The aim of this study was to establish a protocol for normal skeletal development, growth and survival of zebrafish larvae through live feed-to-microdiet transition at an appropriate rate. Four feeding regimes including feeding exclusively on Artemia nauplii (A) or dry microdiet (D), and feeding on both Artemia and microdiet at two different transition rates (slow (B) or fast (C)) were applied from 5 to 24 dpf (days post-fertilization). Results demonstrated a significant effect of feeding regimes on the incidence of skeletal abnormalities (gill cover, fins and vertebral column, $p < .05$) in zebrafish larvae. The A and B experimental groups presented the highest (88 ± 3 and 84 ± 17%, respectively), but the C and D the lowest (18 ± 14 and 11 ± 2%, respectively), rates of normal fish (fish without any abnormality). Similarly, growth rate was comparatively elevated in A and B groups. No significant differences were observed in fish survival between A, B and C groups. However, D group presented a significantly lower survival rate. To our knowledge, this is the first study to show that the live feed-to-microdiet transition rate influences larval growth, survival and abnormality rates in a non-homogenous pattern.

Keywords : feeding, fish larvae, skeletal abnormalities, welfare

1. Introduction

Skeletal abnormalities have been frequently reported to develop in reared fish, as a result of unfavorable environmental conditions and often under the control of genotype (Boglione et al., 2013; Fragkoulis et al., 2017; Fragkoulis et al., 2019). They develop mainly during the embryonic and larval period, up to the end of skeleton ontogeny and rarely in the following developmental stages. In addition to their significant effects on fish morphology, they may also influence fish survival, growth, swimming mode or susceptibility to diseases (Boglione et al., 2013; Koumoundouros, 2010). Hence, the presence of skeletal abnormalities has been suggested as an index of animal welfare, as well as of the appropriateness of husbandry practices applied during the early life (Boglione et al., 2013; Huntingford et al., 2006; Sloman et al., 2019).

Zebrafish is a valuable model species for developmental biology and genetics, fish biology, ecotoxicology, neurophysiology and biomedicine (Bhagat et al., 2020; Lieschke, Currie, 2007; Patterson, Parichy, 2019; Spence et al., 2008), including human skeletal pathology with respect to genetic disorders (Boswell, Ciruna, 2017; Gistelinck et al., 2018). Despite the significance of husbandry conditions for the normal skeleton development of this species, existing relevant literature is scarce (Martini et al., 2021; Martins et al., 2019; Printzi et al., 2021). Understudying the importance of nutrition for fish biological performance, as well as for zebrafish maintenance cost and easiness, different studies target on establishing larval feeding protocols for fast growth, high survival rates and improved reproduction capacity (Carvalho et al., 2006; Delomas, Dabrowski, 2019; Gomez-Requeni et al., 2010; Kaushik et al., 2011; Lawrence et al., 2015; Önal, Langdon, 2000), but rarely for normal skeletal development (Martins et al., 2019).

Live food is traditionally used in zebrafish larval rearing either exclusively or partially during first days of exogenous feeding. Existing protocols involve the use of cultured paramecia (*Paramecium* sp.), rotifers (*Brachionus* sp.) or Artemia nauplii (*Artemia* sp.), in different combinations and variable rates of transition to dry commercial microdiets (Best et al., 2010; Lawrence et al., 2015; Markovich, Brown, 2005; Westerfield, 2000). Despite the extensive use of live feed in zebrafish larval rearing, the shift to dry microdiets has been considered as a critical step for research on nutritional preferences of fish larvae, as well as for the establishment of standard larval rearing protocols with low nutritional variability and high biosecurity level (Cahu, Infante, 2001; Watts et al., 2016). Currently, a variety of microdiets has been developed for the larval rearing of zebrafish, with associated feeding protocols rarely been tested with respect to the morphological quality of fish. To our knowledge, in the only existing relevant study, Martins et al. (2019) demonstrated that the early transition from Artemia nauplii to microdiets significantly reduces vertebral abnormalities in zebrafish (from 90% to ca 50%).

In the present paper we examined the effect of live-feed to microdiet transition rate on the prevalence of skeletal abnormalities, growth and survival rate of zebrafish larvae. We tested Artemia nauplii AF grade, which have been shown suitable live feed for first feeding zebrafish larvae (Martins et al., 2019) and one commercial zebrafish diet.

2. Materials and methods

2.1. Maintainance of fish populations

The effect of four different larval feeding regimes on zebrafish skeleton was tested during the early larval period, from first feeding to 7-10 mm standard length (SL,

24 days post fertilization, dpf), where most of the skeletal features have been developed (Bird, Mabee, 2003; Parichy et al., 2009). Feeding regimes comprised of exclusive feeding on Artemia nauplii (A) or dry microdiet (D), as well as combined use of Artemia and microdiet of two different transition rates (slow or fast, B, C) (Fig. 1, Table 1). At the end of the experimental period all groups were examined for the presence of skeletal abnormalities, survival and growth rates. All treatments were applied in triplicate.

For each replicate trial, approximately one thousand embryos were collected and divided into four cubic net pens (4.5 L volume each), of 100 μm mesh size, allowing the free water circulation in the pen, but not the escape of Artemia nauplii. Pens were placed into one common aquarium of 40 L volume, equipped with a closed recirculation system. After swimbladder inflation (5 dpf), fish larvae were fed 5 times daily, with newly hatched Artemia nauplii (Artemia AF, INVE, Dendermonde, Belgium) and/or commercial dry microdiets (Zebrafeed<100 μm, 100-200 μm, 200-400 μm, Sparos Lda, Olhao, Portugal) (Table 1). All experimental treatments were reared under common abiotic conditions, with temperature, oxygen, pH and conductivity being monitored daily (Table 2). Nitrogen compounds were recorded on a weekly basis (Table 2).

Fertilized eggs were obtained from a common broodstock of 250 wild type zebrafish (ZF WT2 F13; Wageningen Agricultural University, Wageningen, The Netherlands), which were kept in a 100 L aquarium (with a closed recirculation system), at 28.0 °C (±0.5 °C), 450–600 μS/cm conductivity, 7.0–7.5 pH, 6.5-7.0 mg/L oxygen concentration and 14/10 h light/dark photoperiod. Breeders were fed twice per day on commercial dry diet (Zebrafeed 400-600, Sparos Lda, Olhao, Portugal), and twice per week on newly hatched *Artemia* nauplii (Artemia AF, INVE, Dendermonde, Belgium).

122 2.2 Skeletal abnormalities, fish survival and fish growth

123 At the end of the trials (24 dpf), all fish were counted and mortality rates were
124 estimated. To estimate the rate of skeletal abnormalities and measure fish body size, a
125 random sample of 52-61 individuals was taken from each population and replicate. Fish
126 were euthanized using an overdose of phenoxyethanol (2-phenoxyethanol, 0.3-0.5
127 ml/L), fixed in phosphate buffered formalin and stained for bone and cartilage (Walker,
128 Kimmel, 2007). Stained fish were photographed under a stereoscope (Olympus SZ61)
129 and measured for standard length (SL, from tip of snout to posterior tip of the caudal
130 peduncle), by means of tpsDig2 software (Rohlf, 2010). In the case of C group (first
131 replicate), sample size was reduced to 30, due to accidental fish loss and thus limited
132 availability. The terminology used to describe cranial, vertebral and fin abnormalities
133 followed Koumoundouros (2010). Multiple abnormality scores were included in the
134 data when an individual presented more than one abnormality types.

135 Differences in abnormality rates between the different treatments were tested by
136 means of G-test (Rohlf, Sokal, , 1981). Kruskal-Wallis and Mann-Whitney tests were
137 used to test the differences in survival rate between the different treatments. ANOVA
138 and *a posteriori* Bonferroni tests were used to test the differences in body size between
139 the different treatments (IBM SPSS Statistics for Windows, v26, Armonk, NY). In all
140 cases, the significance level was se to 5% (a= 0.05).

141

142 2.3 Feed proximate analysis

143 Chemical analyses of feeds were performed in duplicate for each sample (Table 3):
144 ash (7 h at 550 °C), crude fat (Folch et al., 1957), and crude protein (Kjedhal method,
145 N×6.25). Phospholipid and neutral lipid contents in the diets were assayed by the
146 Juaneda and Rocquelin (1985) method.

147

148 2.4 Ethical statement

149 All the experimental procedures involving zebrafish were performed in accordance
150 with Greek (PD 56/2013) and EU (Directive 63/2010) legislation for animal
151 experimentation and welfare. All protocols were approved by the Animal Care
152 Committee of the Biology Department of the University of Crete (Permit number:
153 81843/20).

154

155 **3. Results**

156 A variety of different abnormality types was observed on the fins, vertebral
157 column and gill-cover of the examined samples. Gill-cover abnormalities were
158 characterized by an inside folding of the operculum and twisted branchiostegal rays
159 (Fig. 2a, 2a'). Fin abnormalities consisted of missing caudal-fin rays and abnormalities
160 of the caudal-fin supporting skeleton (Fig. 2b), as well as of abnormal formation of anal
161 or dorsal-fin pterygiophores and their associated rays (Fig. 2c). Observed vertebral
162 abnormalities involved pre-haemal kyphosis (Fig. 2d), scoliosis of the caudal peduncle
163 (Fig. 2f), or miscellaneous abnormalities (shape and size deformations, missing
164 elements) of vertebrae and their processes (e.g. Fig. 2e).

165 Larval feeding protocol significantly affected the incidence of skeletal
166 abnormalities, with the A and B experimental groups presenting the highest (88±3 and
167 84±17% respectively) and C and D the lowest (18±14 and 11±2% respectively) rates
168 of normal fish (i.e., fish without any abnormality, Fig. 3a). Exclusive feeding of
169 zebrafish larvae on Artemia nauplii (group A) resulted in complete absence of
170 abnormalities in some anatomical areas (i.e. caudal-fin, dorsal fin and gill-cover
171 abnormalities, Fig. 3b, 3d, 3f, 3h), or to negligible abnormality rates in other (Fig. 3c,

3e, 3g). Compared with group A, the slow transition from Artemia to microdiets (group B) did not significantly increase the different abnormality rates (Fig. 3b-3g), except of gill-cover abnormalities which presented a small increase to 12±17% (Fig. 3h). The incidence of fin abnormalities, pre-haemal kyphosis and gill-cover abnormalities significantly increased as Artemia was further replaced with microdiet (groups C and D, Fig. 3b, 3c, 3e, 3h). Caudal-peduncle scoliosis and miscellaneous vertebral abnormalities presented a different response, with comparatively elevated rates at intermediate levels of Artemia replacement (group C, Fig. 3f, 3g).

Larval feeding protocol significantly influenced fish survival at the end of the trials (24 dpf), with group B presenting the highest (89±5%) and group D the lowest survival rate (55±10%, Fig. 4a, 4b). As it was indicated by the larval size (SL) at the end of the trials, feeding regimes significantly affected zebrafish growth rate. Groups A and B presented significantly larger larvae than groups C and D (Fig. 4c).

4. Discussion

Despite the great advances of the last years in microdiet preparation technology and in fish larval nutrition, the rate of transition from live feed to microdiets is still a critical factor for the successful rearing of many species, with significant effects on fish survival, growth and skeletal development (Kestemont et al., 2007; Kolkovski, 2013; Łączyńska et al., 2016). In the present paper we examined the effect of Artemia to microdiets transition rate on the development, survival and growth of zebrafish larvae. Our results showed that differences in survival or growth rates between the regimes with Artemia (groups A, B and C) were insignificant or very small, respectively. When skeletal abnormalities were taken into consideration, the results of the present study

showed that the fast transition to dry feed (groups C and D) has significant negative effects on the skeletal development of the larvae.

No data exist on the effects of different commercial dry feeds on zebrafish skeletal development and thus, we are unable to discuss the effectiveness of microdiets to live feed replacement during early zebrafish feeding. In the only relevant study, Martins et al. (2019) were the first to show significant differences in the abnormality rates between different zebrafish larval feeding protocols, involving exclusively Artemia or the combined use of Artemia and microdiets (experimental or commercial). Reported rates concerned only vertebral abnormalities and were comparatively elevated in the fish fed exclusively Artemia (90% vs 48-51% in the other two groups) (Martins et al., 2019). In the present study, the Artemia group (A) presented the lowest rate of vertebral abnormalities (3.5±3%), as well as of all types of abnormalities recorded (12±3%, vertebral, fins, skull). Observed differences between the two studies could be attributed to the effect of other rearing parameters (e.g. use of static water conditions during the early larval period by Martins et al. 2019), and/or to the genetic background of fish, all of which are well known to have a significant role in the development of skeletal abnormalities (Boglione et al., 2013; Fragkoulis et al., 2019; Fragkoulis et al., 2017). In the present study, the combined use of net pens and a common recirculation system ensured high water- quality conditions, common for all treatments of each replicate throughout the entire experimental period (Table 2).

In most fish species, first feeding larvae are characterized by limited prey-capture capabilities and an immature but rapidly developing digestive system (Zambonino-Infante *et al.* 2008). Thus, successful replacement of planktonic live preys by artificial dry feeds is based on a variety of feed characteristics (e.g. size, sinking rate, color, leaching rate, biochemical composition and ingredients bioavailability, digestibility

etc) and their suitability to match the preferences of developing larvae (Cahu, Infante, 2001; Kolkovski, 2013; Lazo et al., 2011; Yúfera, 2011). Similar to our results, previous studies on other fish species have also shown that the early shift from Artemia to compound diet may result to increased rates of skeletal abnormalities (Kestemont et al., 2007; Łączyńska et al., 2016). This effect could be linked with the difficulty of early larvae to ingest, digest or absorb dry feed, and/or with possible nutritional imbalances of live feed with respect to critical components for bone development (e.g. vitamins, fatty acids, amino acid profile, reviewed by Zambonino-Infante & Cahu, 2010).

Skeletal abnormalities are common in zebrafish laboratory stocks (Murray et al. 2020). In their recent review, with 12-years data from 752 cases (involving 10121 fish), Kent et al. (2020) indicated vertebral (lordosis, kyphosis, scoliosis, platyspondyly) and opercular abnormalities as of the most important and common non-infectious diseases in zebrafish research facilities. In the present paper, a variety of skeletal abnormalities was shown to develop in the zebrafish skull (i.e. gill-cover abnormalities), fins (e.g. abnormal caudal-fin) and vertebral column (e.g. pre-haemal kyphosis, scoliosis of the caudal peduncle). The majority of these abnormalities has been reported to develop in other fish species too, at occasionally high rates (Koumoundouros, 2010). Gill-cover abnormalities are mainly induced by the inward folding of the opercle, subopercle and branchiostegal rays, during the early larval stage (Koumoundouros et al., 1997; Verhaegen et al., 2007). Caudal-fin abnormalities are expressed in a variety of phenotypes, involving lateral bending, duplication, partial lack or stricture (Boglione et al., 2013; Koumoundouros, 2010). In European sea bass, pre-haemal kyphosis was shown to be associated with abnormalities of the branchiostegal rays, and induce high mortality rates due to significant compression of the neural tube (Koumoundouros et

al., 2002). To our knowledge, zebrafish is the only species developing caudal-peduncle scoliosis at high rates (present study, Martins et al., 2019).

In the present study, the response pattern of abnormality rate against larval feeding regime was not uniform for all the reported abnormalities. The rate of scoliosis and miscellaneous vertebral abnormalities was elevated in C group, whereas the rate of rest abnormalities was elevated in D group. As different skeletal elements develop during different ontogenetic periods, which are often characterized by different larval nutritional preferences, the various types of skeletal abnormalities may present variable response patterns against the same factor (Koumoundouros, 2010; Mazurais et al., 2009). Our results on the elevated rates of scoliosis and miscellaneous vertebral abnormalities in C group, might be related with a difficulty of the early larvae (10 dpf) to adapt to a rapid shift in their diet (vs the slower shift in D group).

A variety of behavioral, morphological, functional and physiological indices have been suggested for the evaluation of fish welfare, with emphasis given on the juvenile and adult phases (Huntingford et al., 2006; Sloman et al., 2019). Despite the high susceptibility of fish larvae to a variety of factors causing skeletal abnormalities, the value of the latter as an index for larval welfare has been underestimated. Our results clearly suggest that the rate of skeletal abnormalities is a valuable welfare index, representative of the conformity of husbandry conditions with the preferences of fish during the embryonic and larval stages. Given the effects of skeletal abnormalities on fish growth, survival and function (Chatain, 1994; Georgakopoulou et al., 2010; Koumoundouros et al., 2002; Loizides et al., 2014; Paperna, 1978), once developed, they negatively affect the wellbeing of fish during the following juvenile and adult stages.

Author contributions

A.P., C.K., S.F., A.D., G.G., M.O. contributed to fish sampling and husbandry, A.P., C.K., S.F., G.K. performed analysis of skeletal abnormalities, growth and survival, D.M., J.Z-I. supervised diet analysis, A.P., G.K. analysed the data, A.P., D.M., J.Z-I., G.K. wrote the paper, G.K. conceived the study. All authors reviewed the manuscript.

Conflict of interest

The authors have no conflict of interest to declare.

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Table 1. Feed quantity (per net pen) and feeding frequency in the different experimental treatments. Art. nau., newly hatched Artemia nauplii. d.f., dry microdiet. dpf, days post-fertilization.

dpf	Feed	Treatment A		Treatment B		Treatment C		Treatment D	
		Quantity (per meal)	Daily meals	Quantity (per meal)	Daily meals	Quantity (per meal)	Daily meals	Quantity (per meal)	Daily meals
5-7	Art. nau.	4000	5	4000	5	4000	5	-	-
	d.f. (mg)	-	-	-	-	-	-	50	5
8-9	Art. nau.	8000	5	8000	3	4000	3	-	-
	d.f. (mg)	-	-	50	2	63	2	63	5
10-11	Art. nau.	8000	5	8000	3	-	-	-	-
	d.f. (mg)	-	-	50	2	63	5	63	5
12-16	Art. nau.	12000	5	12000	2	-	-	-	-
	d.f. (mg)	-	-	75	3	75	5	75	5
17-22	Art. nau.	16000	5	12000	1	-	-	-	-
	d.f. (mg)	-	-	100	4	100	5	100	5
23-24	Art. nau.	20000	5	-	-	-	-	-	-
	d.f. (mg)	-	-	150	5	150	5	150	5

422 **Table 2**. Mean values (± SD) of abiotic parameters throughout the three replicates.
423 Oxygen was measured in each net pen separately, whereas rest abiotic parameters were
424 measured in the water outside the pens.

Parameters	Nutritional Treatment	Replicate I	Replicate II	Replicate III
Temperature (°C)	A-D	28.1±0.2	28.0±0.2	28.1±0.3
pH	A-D	7.7±0.2	7.5±0.2	7.8±0.2
Conductivity (μS/cm)	A-D	509±31	516±77	531±55
Ammonia (mg/L)	A-D	<0.01	<0.01	<0.01
Nitrate (mg/L)	A-D	15±14	9±13	4±6
Nitrite (mg/L)	A-D	0.030±0.040	0.014±0.020	0.003±0.008
O_2 concentration (mg/L)	A	6.9±0.5	6.8±0.7	6.7±0.3
	B	6.9±0.5	6.8±0.6	6.6±0.4
	C	6.7±0.4	6.6±0.7	6.5±0.3
	D	7.0±0.4	6.7±0.8	6.7±0.4

425

426

427 **Table 3**. Chemical composition (%) of the experimental diets tested (mean values).

Composition (%)	Artemia nauplii	Dry feed
Dry matter	94.8	91.0
Ash	6.54	12.7
Total Lipids	19.2	13.0
Neutral Lipids	9.2	4.0
Phospholipids	6.6	6.3
Proteins	61.7	67.7

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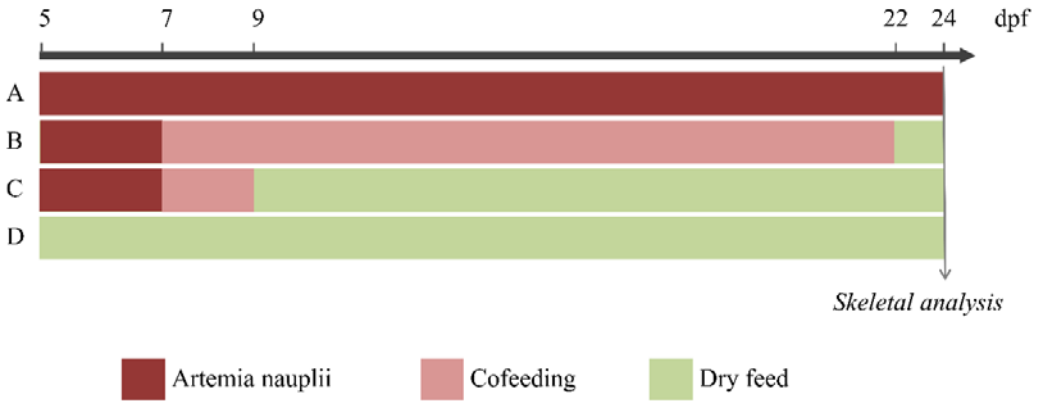
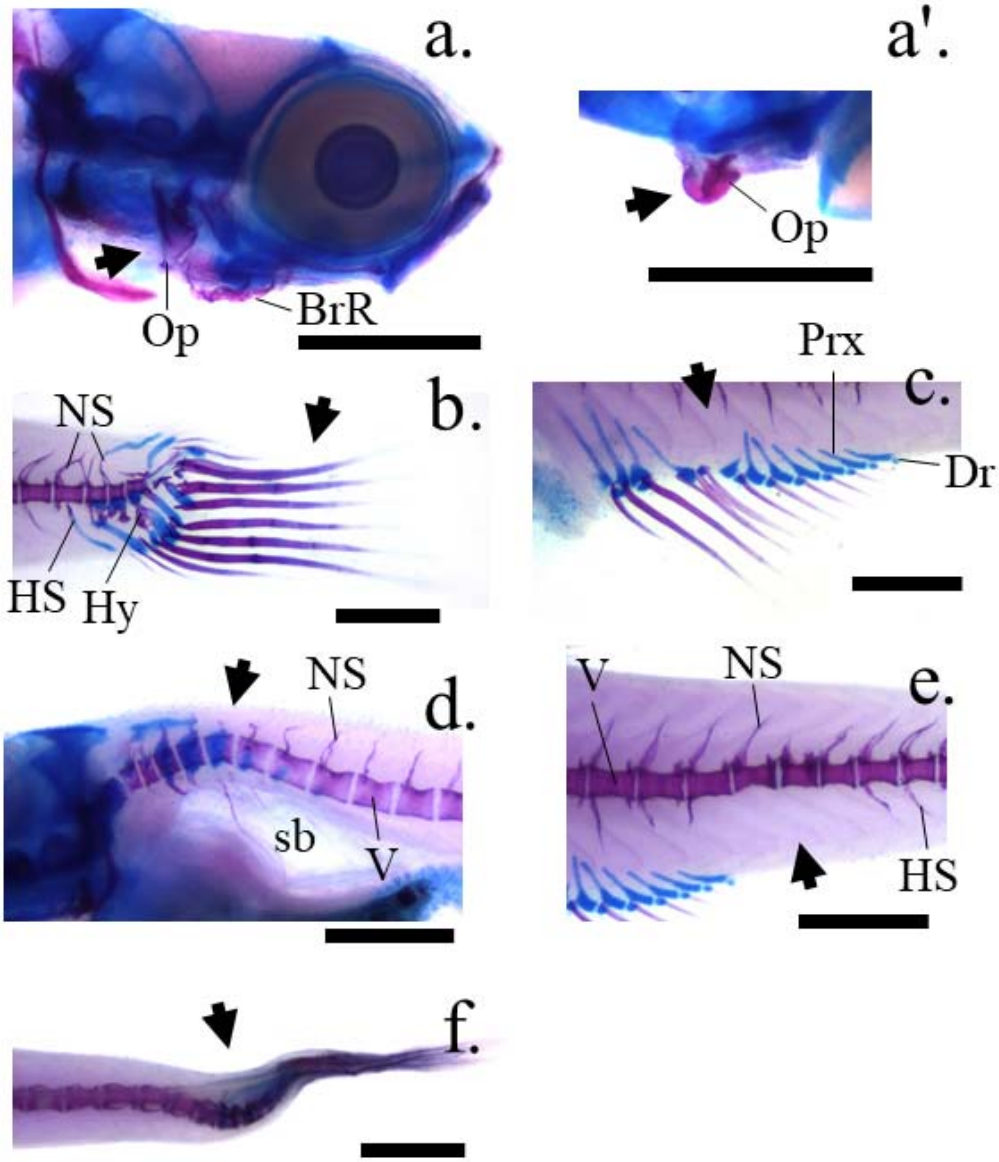
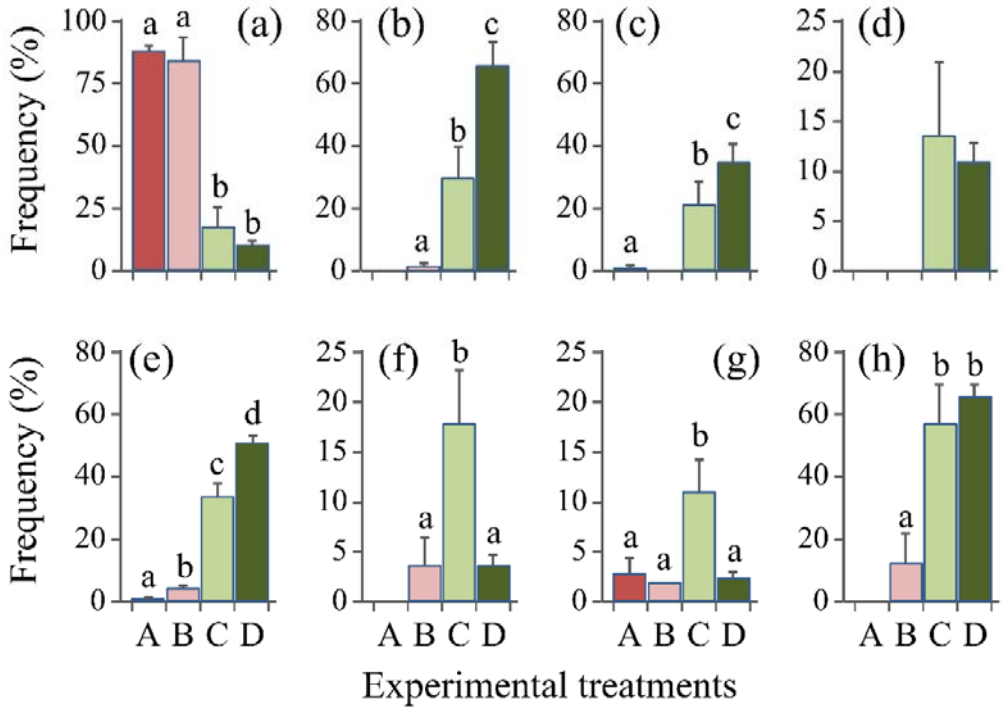


Figure 1. Experimental design. Fish were reared under common abiotic conditions, divided in four feeding regimes including Artemia nauplii (A), dry feed (D) and co-feeding of both (B, C). dpf, days post fertilization. Feed quantities and feeding frequencies are given in Table 1.



436

437 **Figure 2**. Main types of skeletal abnormalities developed in zebrafish larvae. (a-a') side
438 and top view of an abnormal gill cover. Arrows show the inside folded operculum. (b)
439 incomplete formation of caudal-fin. (c) abnormal anal fin, with lacking pterygiophores
440 (arrow). (d) pre-haemal kyphosis. (e) abnormal vertebrae with missing haemal
441 processes. (f) scoliosis of the caudal peduncle. (BrR) branchiostegal rays. (Dr) distal
442 radial. (HS) haemal process. (Hy) hypural. (NS) neural process. (Op) operculum. (Prx)
443 proximal pterygiophore. (Sb) swimbladder. (V) vertebral centrum. Scale bars = 0.5 mm.



444

445 **Figure 3**. Effect of larval feeding protocol (A-D) on the frequency of skeletal
446 abnormalities in zebrafish larvae. (a) Normal fish (fish without any abnormality). (b)
447 Caudal-fin abnormalities. (c) Anal-fin abnormalities. (d) Dorsal-fin abnormalities. (e)
448 Pre-haemal kyphosis. (f) Scoliosis of the caudal peduncle. (g) Miscellaneous vertebral
449 abnormalities. (h) Abnormal gill-cover. Different letters indicate significant differences
450 ($p<0.05$). Error bars equal to 1 SE.

451

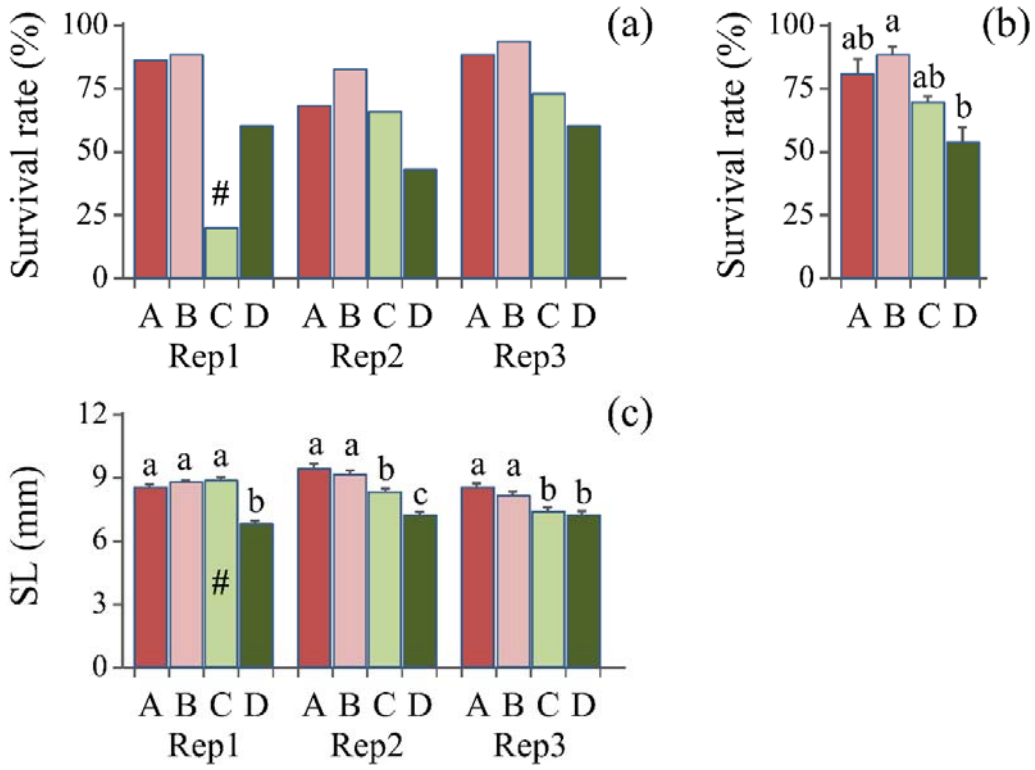


Figure 4. Effect of larval feeding protocol (A-D) on the survival and growth of zebrafish larvae. (a) Survival rate of the different protocols and replicates (Rep1-Rep3). (b) Mean survival rate in the different protocols. (c) Mean standard length (SL) of zebrafish larvae in the different protocols and replicates (Rep1-Rep3), at 24 days post-fertilization. Different letters in (b) and (c) indicate significant differences ($p<0.05$). # indicates the accidental loss of larvae in C group of the first replicate (Rep1). This group was excluded from the mean estimation in graph (b). Error bars equal to 1 SE.