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Comparison of dietary phospholipids and neutral lipids on skeletal development and fatty acid composition in Atlantic cod (*Gadus morhua*)

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Abstract:

Dietary essential fatty acids may affect larval skeletal formation. The aim of this study was to compare effects on growth and osteological development of dietary docosahexaenoic acid (DHA) and eicosapentaenoic acid (EPA) being incorporated in the phospholipid (diets PL1 and PL3) or in the neutral lipid (diet NL1) fraction of the larval diet for Atlantic cod (Gadus morhua). The diets were labelled according to the estimated percentage of total n−3 fatty acids contained in the dietary neutral lipid (NL1-1.3% of dietary dry matter) or in the phospholipid fraction (PL1-1.1% and PL3-2.3%). Larvae were weaned to the isoenergetic and isolipidic microdiets from 17 days post hatching (dph). They were co-fed enriched rotifers until 24 dph and received thereafter only the experimental diets until 45 dph (at 12 °C).

Dry weight on 45 dph was significantly higher in the PL1- and PL3-larvae (mean 2.74 ± 0.2 mg DW) than in the NL1-larvae (mean 2.17 ± 0.1 mg DW). Larvae fed the PL3-diet had a significantly higher DHA content than larvae from the other treatments. No differences were found in larval content of EPA and arachidonic acid (ARA). Larvae fed both PL-diets showed a significantly faster ossification of the vertebral column compared to larvae fed the NL1-diet, according to both larval size and age. On 45 dph, PL3-larvae also had a significantly higher number of fin rays than the other groups at comparable larval sizes. Deformities (mainly kyphosis and shortened vertebrae) were observed in 11% (all groups) of the larvae on 45 dph, with no significant differences among the groups.

Ossification of the vertebral column in the cod larvae was first observed in the neural arches on the anterior part of the spine (21 dph, > 6.9 mm standard length, SL), followed by the vertebrae (25 dph, > 7.9 mm SL), haemal arches (31 dph, ≥ 8.4 mm SL) and parapophysis (35 dph, > 8.7 mm SL). Ossification of all vertebral elements was observed in 45-day-old larvae ≥ 11.4 mm SL. Large variations between larvae in number of ossifying structures between 31 and 35 dph demonstrated that this was the period of most rapid skeletal change (size range 8.2–11.4 mm SL), and that the ossification process was more susceptible to fatty acid lipid source rather than to quantity of dietary fatty acids. We suggest that an optimal dietary content of n−3 HUFA in feed for cod larvae is higher than in the PL1-diet.

Keywords: *Gadus morhua*; Cod; Fish larvae; Osteological development; Skeletal deformities; Dietary requirements; Phospholipids; Fatty acids; EPA; DHA

1. Introduction

Atlantic cod (*Gadus morhua* L.) has an increasing importance in commercial cold water marine aquaculture, and the production of high quality juveniles is still a bottleneck. One of the problems in hatchery reared cod is the presence of skeletal malformations, especially those affecting the anterior part of the vertebral column. Skeletal malformations in cod juveniles from commercial hatcheries have varied between 25 - 85% (Lein *et al*. 2006), and this has negative implications for fish welfare and is causing economic losses to the producers.

Malformations in fish may partly be attributed to suboptimal larval nutrition (Lall and Lewis-McCrea 2007). Nutritional factors (Cahu *et al*. 2003a, b), rearing conditions (Daoulas *et al*. 1991; Andrades *et al*. 1996; Boglione *et al*. 2001; 2003), and genetics (McKay *et al*. 1986, Sadler *et al*. 2001; Kolstad et al. 2006) play essential roles in the skeletal development of fish larvae and may be correlated to abnormalities. Deficiencies in dietary components such as essential fatty acids, phospholipids, amino acids, peptides, vitamins and minerals (Kanazawa *et al*. 1981; Tacon 1992; Gapasin *et al*. 1998; Cahu *et al*. 2003a; b) can interfere with the normal development of larvae, affecting fry quality. However, relatively few studies have been carried out on nutritional requirements in first feeding cod (Rosenlund and Halldorsson 2007).

Few studies have been related to the role of essential fatty acids in marine larval bone development (Cahu et al., 2003b; Villeneuve et al., 2005). Bones of marine fish may contain high concentrations of lipids (Phleger, 1991), and with relatively higher proportions of PUFAs than in mammals. The HUFA requirements of marine fish vary both qualitatively and

quantitatively (Rainuzzo et al. 1997; Sargent *et al*. 1999; Izquierdo, 2004), and the optimal level of EPA and DHA in marine finfish larvae seem to be about 3% of dietary dry matter (Cahu and Zambonino Infante, 2001; Sargent et al., 2002). Cod larvae seem to have relatively high requirements for DHA and EPA, especially in the earliest days of feeding (Galloway et al., 1999; O`Brien-MacDonald et al. 2006).

Most studies determining HUFA requirements in fish larvae have been conducted with live prey enriched with fish oils, where EPA and DHA are mainly incorporated in the dominating triacylglycerides (TAG) (Izquierdo *et al*. 2000). Thus, the effect of lipid classes, such as phospholipids (PL) and neutral lipids (NL) is little studied, although dietary phospholipids are essential for larval development (Coutteau et al. 1997; Cahu et al. 2003b). Cahu *et al*. (2003a) found that growth and skeletal deformities during early development in sea bass (*Dicentrarchus labrax*) were more linked to the proportions of dietary PL and NL than to the total dietary lipid content. Furthermore, Gisbert *et al*. (2005) concluded that dietary PL was more efficient than NL for PUFA supply in European sea bass larvae.

Despite the growing interest in cod, only few studies have described osteological development in cod larvae (Morrison 1993; Grotmol et al. 2005), and there are no controlled, experimental studies of the skeletal development related to larval size or dietary factors in cod. Understanding of osteological ontogeny is important to eliminate abnormal development under rearing conditions, as this may be induced as early as during the embryonic and larval stages (Daoulas *et al*. 1991). The notochord, which constitutes the main axial support during embryonic and early larval stages, begins at the neurocranium and ends at the root of the tail fin. Ring-shaped mineralized zones, known as chordacentra; develop segmentally within the notochordal sheath, forming the initial anlagen of the vertebral bodies (Grotmol *et al*. 2003;

Fleming et al. 2004). Then, sclerotomal osteoblasts condense around the notochord on the surface of the chordacentra, utilising them as foundations for further growth of the vertebrae through direct ossification. The complete vertebral column is formed by the vertebral bodies with their typical hour-glass shape together with other sclerotomal structures, such as neural and haemal arches and the parapophysis (Harder 1975).

The aim of the present study was to compare effects on growth and osteological development of dietary DHA and EPA being incorporated either in the phospholipid (PL) or in the neutral lipid (NL) fraction of the larval feed for Atlantic cod (*Gadus morhua*), and to study effects of different n-3 HUFA levels in the dietary PL fraction. The experimental design was based on cod larvae fed isolipidic and isoproteic microdiets from 17 dph until 45 days post hatch.

2. Materials and methods

2.1 Larval rearing.

Atlantic cod eggs were received 2 days before hatching from a commercial hatchery. They were disinfected in a seawater solution of 400 ppm glutaraldehyde for 5 minutes (Salvesen and Vadstein 1995) before transfer to 160 l cone bottomed tanks at densities of 150 egg l^{-1}. After hatching, the temperature was gradually increased from 7.5 to 12°C within 6 days and was maintained at 12°C ± 0.2°C throughout the experiment. Water exchange was gradually increased from 0.1 l min^{-1} to 0.9 l min^{-1}, and the larvae were reared under continuous light conditions (40 W bulbs).

Rotifers were cultivated for five days in 20 ppt sea water at 20ºC, before starting a daily 20% dilution rate of the cultures (Olsen et al., 1993; Olsen, 2004). The rotifers were long term-enriched with Baker's yeast supplemented with 10 % of the marine lipid emulsion "Marol E" (produced by SINTEF Fisheries and Aquaculture, Norway; Wold et al., 2008a).

Cod larvae were fed rotifers three times every day from 3 dph. The feed density was 3000 – 5000 individuals L^{-1} until 4 dph, and then increased to 5000–7000 individuals L^{-1} from 5 dph. Algal paste (Nannochloropsis sp.; Reed Mariculture, Campbell, California, USA) was added from 2 dph until the end of the rotifer phase in a concentration of 2 mg Carbon L^{-1} three times a day. From 17 to 24 dph, there was an overlap between rotifers and formulated diets with a gradual reduction in the amounts of rotifers. From 24 dph, cod larvae were fed formulated diets exclusively. Small amounts of the formulated diets were fed manually to the larvae (0.15 g x 10) on day 17 to start weaning. On 18 dph, 3 g day^{-1} of formulated diets were added using continuous automatic belt feeders, gradually increasing the amount of feed added per day to each tank to 10 g from 31 dph. A pellet size of <200 lm was used from 17 to 30 dph with an increase to 200–400 lm from 30 dph. An overlap using a mixture of both pellet sizes was conducted from 30 to 36 dph.

Each treatment was run with three replicate tanks. Mortality was registered daily from 18 dph. The experiments and treatment of all larvae were conducted according to the Norwegian "Animal Protection Law".

2.2 Experimental treatments.

Three isoproteinic and isolipidic diets, which varied in lipid class composition were used (Table 1; see also Gisbert *et al.* 2005). The diets were labelled according to the estimated percentage of n-3 fatty acids contained in the neutral lipid (NL1) or in the phospholipid

fraction (PL1 and PL3) of the diets. In the PL3 and PL1 diets, marine lecithin was incorporated in two different levels into the diets in an inverse relation to soybean lecithin, whereas NL1 contained only soybean lecithin as the PL source and marine TAG (cod liver oil) (Table 1). PL1 and NL1 diets had comparable EPA and DHA levels, either in the phospholipid (PL1) or in the neutral lipid (NL1) fraction. The PL3 diet contained twice the amount of EPA and DHA in the PL fraction compared to PL1 (Table 1). The diets were manufactured by UMR 1067 of Fish Nutrition IFREMER (France). The diets were similar to those used by Gisbert et al. (2005), and further details of diet production, composition and analyses of diet for lipid, protein, and ash content is described in that publication (op. cit.). Feed particle sizes were < 200 µm and 200 – 400 µm.

2.3 Sampling.

All larval sampling was carried out randomly from the tanks. The sampled larvae were anaesthetized in 3-aminobenzoic acid ethyl ester (MS222, Tamro 257675) and rinsed in freshwater before further treatment.

For dry weight (DW), 15 larvae were sampled per tank on 1, 10, 17, 24, 35, and 45 dph. Larvae sampled for dry weight were transferred individually to pre-weighed lightweight tin capsules, dried at 60° C for 48 h and weighed on a micro balance (Mettler Toledo microgram balance UMX2,max 2.1 g, d= 0.1, Switzerland).

For fatty acid analysis, 15 larvae were sampled from all tanks on 17 dph, and 15 larvae per tank were sampled on 24, 35 and 45 dph. The samples were flushed with N_2 and kept frozen at -80 °C.

For osteological development and malformation incidences, 10-15 larvae were collected per treatment (1, 6, 10, 13, 17, and 21 dph) and per tank (25, 31, 35, and 45 dph). They were fixed in 10% formalin in phosphate buffered saline (pH 7.4, Apotekerproduksjon AS, Norway) and stored at 4°C.

2.4 Growth and survival

The larval specific growth rate (SGR) and the daily weight increase (DWI in % per day) was calculated according to Kjørsvik et al. (2004):

$$SGR = \ln(W_t/W_0)/t \quad (1)$$

where W_0 is the initial individual dry weight and W_t is the individual dry weight at time t.

$$\% \text{ DWI} = (e^{SGR} - 1) \times 100 \quad (2)$$

Larval survival was calculated by daily counting of dead larvae from 17 dph, and by counting remaining larvae at the end of the experiment. Numbers were corrected for sampled larvae.

2.5 Fatty acid analyses of diets and larvae

Lipid content of eggs and diets was extracted by use of a modified method of Bligh and Dyer (1959) and total lipids were determined gravimetrically according to Rainuzzo et al. (1992). An internal standard 19: 0 methyl ester (Nu-Chek Prep, U.S.A) was added to the samples before extraction. Fatty acid methyl esters (FAMEs) were prepared using the method of Metcalfe et al. (1966) and modified by Rainuzzo et al. (1992). FAMEs were quantified by a capillary gas chromatograph (Perkin Elmer, AutoSystem XL, USA) equipped with a PPC

195 flame ionization detector and WCOT Fused Silica, coasting CP-Wax 52 CB capillary column
196 (Chromopack, the Netherlands). The gas chromatograph oven was set to increase from an
197 initial 90 ^{o}C to 150 ^{o}C at the rate of 30 ^{o}C/min, and thereafter at 3 ^{o}C/min to the final
198 temperature of 225 ^{o}C. Helium was used as carrier gas. The fatty acids were identified
199 according to a commercial standard (68 D, Nu-Chek Prep, U.S.A) and quantified according
200 to the response compared to the internal standard. Two replicates of of each diet and three
201 replicates per treatment of the cod larvae were used to determine total lipid and fatty acid
202 composition
203

204 ***2.6 Histological analyses.***

205 For analyses of bone ossification and osteological abnormalities, 10-20 larvae from each
206 treatment and sampling day were stained in alizarin red (C. I. 58005, Sigma, USA), according
207 to Balon (1985). Fixed larvae were rinsed twice in distilled water for 5 min, dehydrated in
208 ethanol (95%, 75%, 40%, and 15%, 30 min each) and rinsed in distilled water for 30 min. To
209 remove pigments, larvae were bleached until transparent in a 1:9 ratio of 1% KOH and
210 hydrogen peroxide (30% H_2O_2, 10 min to 2h) under strong illumination. Larvae were then
211 cleared using trypsin (T7409, Sigma, USA) dissolved in 0.3% borate buffer for up to 20h until
212 all muscle tissue was transparent. Following trypsin treatment, larvae were stained for
213 calcified structures by immersion in a 1% KOH solution with a few drops of an alizarin red
214 solution to obtain a deep purple colour. Two photos of each larva were taken before the larvae
215 were individually stored in plastic trays in 100% glycerine with a few crystals of thymol
216 (gradual transfer to 40, 70 and 100% glycerine).
217

218 The remaining fixed larvae were stained in Alcian blue (8GS(8GX), USA) and Alizarin red
219 following the staining procedure used by Balon (1985), with the addition of a neutralization

step to increase the pH after staining in Alcian blue (after Gavaia *et al*., 2000). The 45 dph larvae from this group were analysed for fin ray development (18-22 larvae per treatment). All stained larvae were examined for occurrence of osteological abnormalities.

Observations were made with a stereo light microscope (Leica MZ75; Germany) equipped with a Sony CCD-IRIS colour video camera (model DXC-107P, Sony, Japan). The standard length (SL, from the tip of the snout to the end of the notochord), and myotome height (MH, muscle thickness behind the anus) were measured by an ocular micrometer in fixed larvae prior to staining. Development of skeletal elements were identified, and neural and haemal arches, vertebrae and parapophysis during the ossification process were counted under a stereomicroscope and/or by photo analysis (Scion Image Program, Scion Corporation). This software was also used to perform fin ray counts in 45 dph larvae and measurements of spinal curvature and swim bladder area. Types of skeletal malformations and incidences per treatment in each stage were registered.

2.7 Statistics.

Results are given as mean values and standard error. Survival and malformation rates were arcsin transformed. Dry weight, fatty acid composition, standard length, number of spinal column elements undergoing ossification, fin ray counts, swim bladder size and incidences of malformations were tested for homogeneity of variances using a Levene test. To compare means, the group data were statistically tested using one-way ANOVA followed by a Student–Newman–
Keuls-Test. When variances were not homogenate; a non parametric Kruskal–Wallis test was accomplished.

To test a potential difference in the ossification process between the treatments, a forward-stepwise regression was used to find the best sigmoidal fit to the larval standard length of the three groups. Expected values were calculated using the sigmoidal, and the residuals were calculated as the difference between the expected and observed value. The residuals of ossifying elements were compared between the three feeding groups, using using one-way ANOVA followed by a Student–Newman–Keuls-Test. If significant differences were not found, larval ossification process was grouped according to larval standard length intervals (6.5 – 7.49 mm, etc) and was tested for differences using one-way ANOVA followed by a Student–Newman–Keuls-Test. Differences and effects were considered significant at $P < 0.05$ for all tests. All statistical analysis was performed using the software SPSS 15.0 for Windows.

3. Results

3.1 Larval growth and survival

At the end of the experiment (45 dph), the mean dry weight of PL3- (2.67 $\pm$ 0.7 mg) and PL1-larvae (2.78 $\pm$ 1.4 mg) were significantly higher than for the NL1-larvae (2.17 $\pm$ 0.1 mg), as showed in Figure 1. The mean daily weight increase (DWI) for the experimental period 17 – 45 dph was 9.1 - 10.1 % per day for all treatments, and between 8.3 – 8.9 % per day from hatching and to 45 dph, with no significant difference between the groups (Table 2). Survival during the dry feed period did not differ significantly between treatments on 45 dph, being 12.2 ± 0.5 % for PL3-larvae, 12.8 ± 3.5 for PL1-larvae and 15.7 ± 4.5 for NL1-larvae.

3.2 Fatty acid composition

The PL3 diet (Table 3), which contained the highest amount of marine lecithin, had a significantly higher content of DHA than the other diets (15% of total fatty acids), whereas the EPA-content of diet NL1 was higher than in any of the other diets. The DHA/EPA ratio of PL-diets was 1.8-2.0, with a lower value of 1.1 for the NL1-diet, and the EPA/ARA ratio was higher in NL1 (10.2) compared to the PL-diets (6.8 - 7.3).

The fatty acid composition of the cod larvae on 45 dph reflected the dietary content (Table 3). Larval DHA-content (Figure 2a) at the end of the rotifer stage (17 dph) was about 15 mg/g dry weight (19% of total fatty acids). The DHA content increased significantly up to 45 dph in the PL3-group, and the PL3-larvae had a significantly higher DHA-content than the other larvae on both 35 and 45 dph. No differences in DHA-content was found between the PL1- and NL1-larvae, and no quantitative differences were found between any larval groups in EPA- or ARA-content at the end of the experiment (Figure 2 b,c).

The DHA/EPA ratios were 3.8 – 3.9 in the PL-larvae, and 2.5 in larvae fed the NL1-diet, and EPA/ARA ratios were 5.9 for the NL1-larvae and 4.3 – 4.6 in PL-larvae (Table 3). Larvae fed NL1- and PL1-diets had higher contents of n-6 HUFAs than the PL3-larvae, and the ratio of n-3/n-6 was significantly higher for PL3-larvae than for NL1- and PL1-larvae (Table 3).

3.3 Ossification of the spinal column elements

In larvae sampled for bone development, no differences were found in mean larval standard length on any sampling day between the dietary treaments (Table 4). The first signs of vertebral ossification was observed in 21 day old larvae from all treatments (Table 4), and ossification of all vertebral elements and fin rays were observed on 45 dph in the largest larvae in all treatments (Fig. 3).

An overview of the age related ossification and mean size of larvae is given in Table 4. The first elements of the vertebral column to undergo ossification were the neural arches. Ossification of these structures was observed from 21 dph. Ossification of the vertebrae began in larvae ≥ 7.8 mm SL from 25 dph, and ossification of haemal arches was first observed in 31 dph larvae (≥ 8.1 mm SL). The ossification process increased rapidly between 25-45 dph, and large variations in numbers of ossifying neural and haemal arches and vertebrae were especially found in all larval groups on 31 and 35 dph. On 31 dph, the mean SL of stained larvae from all treatments were similar (8.4-8.5 mm), and PL3-larvae had a significantly higher mean number of ossifying neural arches and vertebrae than the NL1-larvae, with PL1-larval values in between these groups (Table 4). A marked age related delay in ossification was thus observed in the NL1-larvae. After 31 dph, no difference was found in age related ossification of the vertebral column elements (Table 4).

A size related comparison of the development of ossifying structures demonstrated a size dependent and rapid increase in ossification of larvae > 8 mm SL for all treatments (Fig. 4). Ossification of neural arches was observed in larvae from 6.9 – 8.6 mm SL, and all fifty pairs could be observed in larvae from 11.1 mm SL (Fig. 4a). Normal larvae had straight vertebrae during the ossification process, and ossification of all fifty vertebrae was observed in the largest larvae (≥ 12.0 mm SL) from all treatments on 45 dph (Table 4; Figs 3 and 4b). Ossifying haemal arches were observed from 8.1 mm SL, and complete ossification of all 31 haemal arches was observed in larvae ≥ 10.6 mm SL (Fig. 4c).

The best fit curve found was the Gompertz growth curves (Fig. 4), which also demonstrated a tendency of higher variation and slower size related ossification of all vertebral elements in

317 the NL1-larvae compared to PL-larvae. While 90 % of PL-larvae reached forty ossified
318 neural arches at 9 mm SL, the same was observed in NL-larvae only from 12 mm length (Fig.
319 4a). Likewise, 90 % of the PL-larvae underwent ossification to forty vertebrae at a range of
320 8.2 – 11.4 mm SL, whereas larvae fed NL1 diet reached this stage at the size interval of 8.4 –
321 11.8 mm SL (Fig. 4b). Larvae in the PL-groups reached ossification of 11 haemal arches
322 mainly in larvae from the PL groups at 8.2 – 9.7 mm SL, whereas the same stage was seen in
323 a much wider size range in larvae fed NL1 (8.2 – 11.4 mm SL, Fig. 4c). No significant
324 differences in the ossification process were found between PL- and NL-larvae using the
325 residuals of ossifying elements in the Gompertz models ($p>0.05$), mainly due to the large
326 variation in larvae from the NL1-group.
327
328 Comparing development of ossifying vertebral elements in larval size intervals (Fig. 5)
329 demonstrated further the tendency to slower size related ossification in the NL1-larvae,
330 especially between 8.5 – 11.5 mm SL. However, large individual variations were observed,
331 and only ossification of the haemal arches showed a significantly slower development in
332 NL1-larvae than in the PL-larvae ($p<0.05$; Fig. 5c).
333
334 Ossification of parapophysis was first observed in 35 dph larvae (> 8.7 mm SL) reaching full
335 ossification in the largest 45 dph larvae (Table 4). No significant differences were found
336 between the diets.
337
338 Development of fin rays on 45 dph (Fig. 3) was significantly more advanced in larvae fed
339 PL3 compared to PL1 and NL1 larval groups (Table 5). The fin ray development was
340 correlated to the larval size in all treatments, and small larvae from all groups had lower
341 number of fin rays than bigger larvae (Pearson correlation, $p<0.01$). However, the PL3 larvae

had a higher number of fin rays than the other groups at comparable larval sizes ($p<0.05$). In the NL1 group, the caudal and 1st dorsal fins had the most variable ray numbers. Some of the larvae from this group had not yet developed rays in the 1st dorsal fin, and at the end of the experiment the NL1 larvae also had lower mean caudal fin ray numbers compared to the larvae fed PL.

3.4 Incidences of malformations and types.

No significant difference was found between the groups with respect to the incidences of malformations at any sampling day. No skeletal abnormalities were observed on 1, 10 and 13 dph, and very low malformation rates were seen until 35 dph (Fig 6a). Skeletal malformations were found in 11 % (all groups pooled) of the larvae on 45 dph, where kyphosis and shortened vertebrae in the anterior part of the spine accounted for 81% of the total observed malformations (Figs. 6b, 7a, 7b). Very few larvae with scoliosis and lordosis (Figs. 6b, 7b), and only one larvae with cephalic malformation (jaw deformity) were observed. In addition to spinal malformations, deformities of fin rays and pterygiophores were found in 6 % of the larvae on 45 dph.

Vertebral curvature (angle) of the larvae was positively correlated to the number of shortened vertebral segment bodies ($p< 0.01$), with no significant differences between the diets. No correlation was found between vertebral curvature (deformed larvae) and swim bladder size ($p>0.05$). The relation between SL and MH was positively linear for both categories of larvae (normal larvae $R^2=0.7759$ and $p<0.0001$; abnormal larvae $R^2=0.6480$ and $p< 0.0003$). The MH was not significantly different between normal and abnormal larvae ($p>0.05$).

4. Discussion

The most important aim of this experiment was to compare possible effects of essential dietary fatty acids being incorporated in different lipid classes. We found that the n-3 HUFAs were clearly more beneficial to larval growth and development when incorporated in the polar phospholipid fraction (PL) than in the neutral lipid (NL) fraction, as demonstrated by higher average dry weight of PL3- and PL1-larvae at the end of the experiment. Larvae fed PL diets also showed a significantly faster ossification of the vertebral column compared to larvae fed the NL diet, when measured both by larval size and by larval age. There was no significant difference in spinal ossification between larvae from the PL1 and PL3 groups. In spite of the difference in growth and skeletal development between NL1- and PL1-larvae, there were no differences in the larval content of DHA, EPA, or ARA between these two larval groups. The larval fatty acid composition clearly reflected the dietary composition, and the only major difference in larval fatty acid composition was the much higher DHA content of PL3-larvae at the end of the experiment. All diets contained the recommended value of 10% phospholipids and n-3 HUFA contents were above that generally required in larval diets (Sargent et al. 1999). Phospholipids constituted about 60-75% of the total lipid content in our diets, which is in the same range as found in copepods (van der Meeren et al., 2008). The copepods that are the most important natural larval diet seem to have a DHA-content ranging from about 4 mg/g DW (van der Meeren et al., 2008) and up to 15-20 mg/g DW (Evjemo et al., 2003), and our experimental diets were within the same range. The DHA/EPA-ratios of these copepods varied between 1.2 – 2.8 (*op. cit.*), which shows that our diets were in the higher end of this scale. We may therefore conclude that fatty acid metabolism and incorporation into cod larval tissues may not only be partly determined by the levels of fatty acids available from the

391 diet (as reviewed by Sargent et al. 1999), but that it is also strongly affected by the dietary
392 lipid source of the n-3 fatty acids.
393
394 All microdiets used in the present study supported positive larval growth and development in
395 Atlantic cod larvae, and larval growth in our experiment was higher than for other early
396 weaning trials with cod (Baskerville-Bridges and Kling, 2000; Wold et al. 2008a). The larval
397 growth rates we obtained in the present study with very early larval weaning, was aso
398 comparable to previous studies of cod larvae fed enriched cultivated prey (Galloway et al.,
399 1999; Brown et al., 2003; Park et al., 2006; van der Meeren et al., 2007; Garcia et al., 2008).
400 However, the larval growth presented here was lower than what we recently obtained for cod
401 using enriched rotifers and *Artemia* nauplii (Wold et al., 2008a).
402
403 In addition to an improved growth, maturation of the digestive system also occurred more
404 rapidly in the cod larvae fed the PL3 and PL1-diets than in larvae fed the NL1 diet (Wold et
405 al., 2007), and the marine phospholipids used in this study had a beneficial effect on the larval
406 liver histology (Wold et al., 2009).
407
408 The nutritional benefits of n-3 HUFAs being incorporated in the dietary phospholipid fraction
409 was expressed as a faster developmental rate of ossification of the vertebral column and fin
410 rays in PL-larvae than in NL1-larvae, both according to larval size and age, although all
411 groups demonstrated a size-dependent skeletal ossification. By the end of the experiment
412 (45dph, 540 d°), ossification of all spinal elements was observed in the largest larvae in all
413 groups (≥ 12.0 mm SL). Studies of larval malformations only from the end of the experiment
414 (45 dph) would therefore not have revealed any differences in dietary skeletal effects (except
415 in number of fin rays).

Cod larvae had a straight notochord at hatching (4.4 mm SL), and no developed structures of the axial skeleton were observed. This observation is consistent with previous findings for cod (Grotmol *et al.* 2005) and for other species like barramundi (*Lates calcarifer*) (Fraser *et al*. 2004), common Pandora (*Pagellus erythrinus*) (Sfakianakis *et al*. 2004) and Senegal sole (*Solea senegalesis*) (Gavaia *et al*. 2002), where the notochord was the only axial suspension structure present at hatching. This feature seems to be characteristic for many larval teleostei.

In our study, the first structures to ossify were the neural arches, followed by vertebrae, haemal arches and parapophysis. The onset of skeletal ossification was observed around 21 – 25 dph (252 –300 d°) dph (size range of 6.9 – 8.6 mm SL). Grotmol *et al*. (2005) also found ossification of the first pair of neural arches in 21 day old cod larvae from commercial hatcheries (fed live prey, 6-11 °C). The main skeletal development of cod larvae in the present experiment seemed to follow a similar pattern and timing of events as observed in the study by Grotmol et al. (2005). However, a direct comparison of our results is not possible, as data of larval size were not given by Grotmol *et al.* (2005). To our knowledge, the present study is the first to describe skeletal development in cod larvae related to the larval size as well as to the age and nutritional quality.

The major spinal ossification process occurred in the size range of 8.2 – 11.4 mm, which mainly corresponded to 31 and 35 days-old larvae. The large variations between larvae in number of ossifying structures between 31 and 35 dph, demonstrated that this was the period of most rapid skeletal change. In this developmental window, the ossification process was more susceptible to fatty acid lipid source rather than to the quantity of dietary fatty acids.

Similar significant differences between treatments in fin ray numbers were found at the end of the experiment, and larvae fed PL3 had significantly higher dorsal, anal, and caudal fin rays number than larvae fed PL1 and NL1 at comparable larval size and age. Fin rays are sensitive to environmental influences, especially to thermal variation (Boglione *et al*. 2001). However, this could not be the cause of fin ray variation found in this study, since larvae from the three treatments had the same rearing conditions, differing from each other only in the microdiets they were fed. The results thus demonstrated that larvae fed the highest level of EPA + DHA in the PL fraction (PL3), developed faster than the other larval groups. A rapid increase in size and development of fin rays should be energetically beneficial for the swimming and predatory ability of cod larvae, due to the length dependence between inertial and viscous forces acting on swimming fish larvae, and to the functionality and effectivity of the larval swimming apparatus (Osse and van den Boogart 1995; 2004; Galloway et al. 1999).

Larval deformities were relatively low in the present experiment (11%) compared to observations from commercial hatcheries, where abnormalities of the vertebral bodies on the anterior part of the spine have been observed in up to 85% of the juveniles (Lein et al., 2006). The high rate of normally developing larvae is probably due to our high quality enriched rotifers, and is further indicating that the different experimental diets did not induce high frequencies of malformations. Cod larvae in our experiment were mainly affected by kyphosis and shortened vertebrae of the anterior part of the spine, while cases of jaw malformation, lordosis and scoliosis were rarely found.

The main types of malformations which were found in the present study were thus in line with similar observations from marine larval hatcheries (Andrades *et al*. 1996; Daoulas *et al.* 1991; Totland *et al*. 2004, Grotmol *et al*. 2005; Lein *et al*. 2006). Multiple variations in rearing

conditions, diets and genetic material will affect spinal deformity rates in cod (Kolstad et al., 2006), which makes it difficult to elucidate the causative factors in these earlier studies with cod. Imsland et al. (2006), found that cod juveniles where larvae had been fed cultivated enriched rotifers had a much higher incidence of caudal spinal deformities than juveniles startfed with natural zooplankton, with 14 and 4% deformities in the two different groups. Similar caudal deformities was not observed in our experiment. However, Imsland et al. (2006) used *Nannochloropsis* for enrichment of rotifers, and these microalgae contain very little n-3 HUFAs compared to other microalgae used in fish larviculture, such as e.g. *Isochrysis galbana* (Reitan et al., 1997). It is therefore possible that the caudal deformities observed in intensively reared cod by Imsland et al. (2006) could have been induced by a lack of essential n-3 HUFAs in the larval feed.

Possible factors related to kyphosis malformations have been reported in a few studies, but with contradictory conclusions. In reared juveniles of sea bass, kyphosis has been related to the non-inflation of the swim bladder, but has also been observed in sea bass larvae with a normal functional swim bladder (Koumoundouros *et al*. 2002). In striped trumpeter (*Latris lineate*), hyperinflation of the swim bladder has been related to kyphosis (Trotter *et al.* 2001). Grotmol *et al*. (2005) observed that this deformity developed as early as 7 dph in cod, during the transition to live feed and two weeks before ossification of the vertebral column components was observed. The critical time window with regard to development of this malformation seemed to be from 18 to 36 dph, when the initial formation of the vertebrae took place, and Grotmol et al. (2005) concluded that the probable causes for kyphosis in cod larvae were an overfilled swim bladder which pressed the notochord upwards. However, such a relation between anomalies of swim bladder and kyphotic larvae was not found in our study. In contrast to the results of Grotmol *et al*. (2005), we observed kyphosis deformities mainly at

491 later stages (35-45 days after hatching). In our study, neither the difference between the diets
492 nor the size of the swim bladder was related to malformations of the vertebral bodies. Thus,
493 an increased swim bladder size can not be the only cause for spinal deformities in cod larvae,
494 although it may be a problem in some larval rearing systems.
495
496 Long-chain n-3 PUFAs seem effective in promoting bone formation in mammals as well as in
497 fish (Lall and Lewis-McCrea, 2007), but these mechanisms are poorly studied in fish. Feeding
498 n-3 PUFAs to growing male rats elevated EPA and DHA, but reduced ARA in various bone
499 tissue compartments , reduced *ex vivo* bone prostaglandin E2 production, and increased rates
500 of bone formation (Watkins et al. 2000). Increasing dietary DHA also lowered the ratio of
501 18:2n-6 (linoleic acid)/n-3 in bone compartments, which favoured bone formation as well as
502 bone conservation in rats (Watkins et al. 2003; 2006). Larvae fed NL1 and PL1 diets
503 contained relatively more n-6 HUFAs than the PL3-larvae, thus increasing the ratio of n-6/n-
504 3. The better bone formation in all PL-larvae compared to NL1-larvae might thus be
505 explained by better availability of the n-3 HUFAs in the PL-diets, as the percentage of n-3
506 HUFAs was quite similar in both the NL1- and PL1-diets.
507
508 The high dietary content of phosphatidylinositol (PI) from 2 to 2.5% (see Table 1) could
509 explain the relatively low malformation rates and that no significant differences were found
510 between the groups in the present study of cod larvae. The content of PI in the three diets
511 were higher than recommended for sea bass (1.6%, Cahu *et al*. 2003a) and carp (*Cyprinus*
512 *carpio*) (1.3%, Geurden *et al*. 1998), where this content resulted in only 2% of deformed
513 larvae. In sea bass larvae, where the same diets as used in the present study, were used from
514 the beginning of exogenous feeding, the PL1 and PL3 diets resulted in good larval growth and
515 survival, with low vertebral deformities, whereas similar levels of DHA and EPA

516 incorporated in the NL were suppressing the growth or were even lethal (Gisbert et al., 2005;
517 Villeneuve et al., 2005). Higher levels of HUFAs also showed that retinoid pathways were
518 negatively influenced by dietary lipids, leading to skeletal malformation in the sea bass larvae
519 (Villeneuve et al., 2005).
520
521 From our cod larval feeding experiment, using the same formulated diets as in Gisbert et al.
522 (2005) as an "*Artemia* replacement", we conclude that the essential n-3 HUFAs were more
523 beneficial for the cod larval growth and skeletal development when they were supplied in the
524 dietary polar phospholipids rather than in the dietary neutral lipids. Larvae fed the PL-diets,
525 with EPA and DHA in the PL fraction, showed a faster ossification than larvae fed n-3 HUFA
526 in the neutral lipid fraction (NL1), and spinal ossification rate and fin ray formation related to
527 larval size were sensitive parameters for dietary osteological effects in cod larvae. Based upon
528 the results from larval fatty acid content and fin ray formation, we suggest that n-3 HUFA
529 requirements in cod larvae is higher than in the PL1 diet. However, further studies should be
530 conducted to find the optimal level of essential HUFAs in the dietary PL fraction for
531 developing cod larvae, and to reveal the mechanisms affected by dietary lipids in larval bone
532 development.
533

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543

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725

Tables

Table 1. Ingredients and proximate composition of the experimental microdiets. The diets were similar to those used by Gisbert et al. (2005), and further details of diet production, composition and analyses of diet for lipid, protein, and ash content is described in that publication (op. cit.).

Diet	**PL3**	**PL1**	**NL1**
Ingredients[a] (g/100 g dry matter)			
Defatted fish meal	51	51	51
Hydrolyzed fish meal (CPSP)	14	14	14
Cod liver oil	0	0	7
Marine lecithin[b]	14	7	0
Soybean lecithin[c]	7	14	14
Vitamin mixture[d]	8	8	8
Mineral mixture[e]	4	4	4
Betain	2	2	2
Proximate composition %			
Proteins (N x 6.25)	61.5	58.3	57.7
Lipids	16.1	16.4	17.9
Phospholipids	12.5	12.4	10.7
EPA + DHA in PL	2.3	1.1	0.3
Neutral lipids	3.7	4.4	6.8
EPA + DHA in NL	0.3	0.3	1.3
Ash	17.4	17.5	17.4
Moisture	7.5	7.1	7.2
Energy (MJ/kg)[f]	16.34	15.92	16.39

[a]All dietary ingredients were commercially available. Fish meal (La Lorientaise, Lorient, France), hydrolyzed fish meal (CPSP, Soluble Fish Protein Concentrate; Sopropêche, Boulogne sur Mer, France), cod liver oil (La Lorientaise), marine lecithin (LC60, Phosphomins™; Phosphotech, Saint Herblain, France).

[b]Contains 60% phospholipids (with 45% PC, 20% Phosphatidil Ethanolamine PE, 16% PI), 5% TAG, 15% cholesterol, and 1 mg/g natural tocopherols as antioxidant.

[c]Contains 95% phospholipids (with 26% PC, 20% PE and 14% PI).

[d]Per kg of vitamin mix: retinyl acetate 1g; cholecalciferol 2.5 mg; all-rac-α-tocopherol acetate 10 g; menadione 1 g; thiamine 1 g; riboflavine 0.4 g; D-calcium pantothenate 2 g; pyridoxine HCl 0.3 g; cyanocobalamine 1 g; niacine 1 g; choline chloride 200 g; ascorbate polyphosphate 20 g; folic acid 0.1 g; biotin 1 g; meso-inositol 30 g; cellulose 732.1 g.

[e]Per kg of mineral mixture: KCl 90 g, Kl_4O 40 mg, $CaHPO_4.2H_2O$ 500 g, NaCl 40g, $CuSO_4.5H_2O$ 3 g, $ZnSO_4.7H_2O$ 4 g, $CoSO_4.7H_2O$ 20 mg, $FeSO_4.7H_2O$ 20 g, $MnSO_4.H_2O$ 3 g, $CaCO_3$ 215 g, $MgSO_4.7H_2O$ 124 g, NaF 1 g.

[f]Calculated as: fat x 37.7 MJ/kg; protein x 16.7 MJ/kg.

748

749 Table 2. Daily weight increase (DWI) (%, means ± s.e., n = 3) for cod larvae during the

750 microdiet feeding experiment (17 – 45 dph) and for the whole larval period (0 – 45 dph).

751

	Cod larval DWI (%)		
Period (dph)	NL1	PL1	PL3
17 – 45	9.1 ± 0.3	10,1 ± 0.6	9,7 ± 0.6
0 – 45	8.3 ± 0.3	8.9 ± 0.2	8.6 ± 0.4

752

753

754

755 Table 3. Lipid and fatty acid content of the experimental microdiets and 45 day old cod larvae at the end of the experiment.

756

	Diets			Cod larvae (45 dph)		
	NL1	**PL1**	**PL3**	**NL1**	**PL1**	**PL3**
Total lipid (mg/g DW)	232.3 ± 2.9[a]	184.2 ± 12.1[a]	183.6 ± 3.5[b]	151.2 ± 8.8	153.8 ± 1.7	148,1 ± 9.9
Sum FA (mg/g DW)	94.3 ± 0.4[a]	67.2 ± 0.6[a]	57.8 ± 0.7[b]	61.6 ± 5.0	64.2 ± 1.2	61,6 ± 1.0
Total n-3 - (mg/g DW)	15.4 ± 0.1[a]	11.3 ± 0.1[b]	15.1 ± 0.5[a]	19.8 ± 0.9[a]	21.0 ± 0.2[a]	26.0 ± 0.3[b]
- (% of total FA)	16.3 ± 0.2[a]	16.7 ± 0.0[a]	26.2 ± 0.5[b]	32.4 ± 1.6[a]	32.6± 0.8[a]	42.2 ± 0.5[b]
Total n-6 - (mg/g DW)	31.7 ± 0.3[a]	27.8 ± 0.0[b]	16.3 ± 0.1[c]	14.0 ± 2.3[ab]	17.1 ± 1.0[a]	9.3 ± 0.4[b]
- (% of total FA)	33.6 ± 0.2[a]	41.3 ± 0.3[b]	28.2 ± 0.2[c]	22.5 ± 2.0[a]	26.6 ± 1.0[a]	15.1 ± 0.6[b]
n-3/n-6 ratio	0.5 ± 0.0[a]	0.4 ± 0.0[b]	0.9 ± 0.0[c]	1.5 ± 0.2[a]	1.2 ± 0.1[a]	2.8 ± 0.1[b]
Saturated FA - (mg/g DW)	24.4 ± 0.0[a]	17.2 ± 0.4[b]	16.6 ± 0.1[b]	17.2 ± 1.0	17.4 ± 0.3	17.0 ± 0.5
- (% of total FA)	25.9 ± 0.1[a]	25.5 ± 0.4[a]	28.7 ± 0.2[b]	28.0 ± 0.6	27.1 ± 0.3	27.5 ± 0.4
Monounsaturated FA - (mg/g DW)	22.8 ± 0.2[a]	11.1 ± 0.1[b]	9.8 ± 0.0[c]	10.6 ± 0.9	8.8 ± 0.1	9.4 ± 0.5
- (% of total FA)	24.2 ± 0.1[a]	16.4 ± 0.1[b]	16.4 ± 0.2[b]	17.2 ± 0.3[a]	13.7 ± 0.2[b]	15.2 ± 0.7[b]
DHA - (mg/g DW)	5.8 ± 0.2[a]	5.0 ± 0.1[a]	8.7 ± 0.5[b]	13.3 ± 0.5 [a]	15.5 ± 0.3 [a]	20.0 ± 0.2 [b]
- (% of total FA)	6.1 ± 0.2[a]	7.4 ± 0.1[a]	15.1 ± 0.6[b]	21.9 ± 1.8 [a]	24.2 ± 0.9 [a]	32.4 ± 0.6 [b]
EPA - (mg/g DW)	5.1 ± 0.0[a]	2.7 ± 0.0[b]	4.4 ± 0.1[c]	5.3 ± 0.4	4.1 ± 0.0	5.2 ± 0.1
- (% of total FA)	5.5 ± 0.0[a]	4.1 ± 0.0[b]	7.5 ± 0.1[c]	8.6 ± 0.1 [a]	6.3 ± 0.1 [b]	8.4 ± 0.0 [a]
ARA - (mg/g DW)	0.5 ± 0.0	0.4 ± 0.0	0.6 ± 0.0	0.9 ± 0.0	0.9 ± 0.0	1.2 ± 0.1
- (% of total FA)	0.5 ± 0.0[a]	0.6 ± 0.0[b]	1.1 ± 0.0[c]	1.5 ± 0.1	1.5 ± 0.1	1.9 ± 0.1
DHA/EPA ratio	1.1 ± 0.0	1.8 ± 0.0	2.0 ± 0.0	2.5 ± 0.2	3.8 ± 0.1	3.9 ± 0.1
EPA/ARA ratio	10.2	6.8	7.3	5.9 ± 0.2	4.6 ± 0.1	4.3 ± 0.1

757 dph, days posthatch; DHA, docosahexaenoic acid; EPA, eicosapentaenoic acid; ARA, arachidonic acid.

758 Values are the mean ± s.e. of two (diets) or three (larvae) replicates, values with different superscript for diets means dietary values are significantly different ($P < 0.05$), larval

759 values with different superscript means larval fatty acid content is significantly different ($p < 0.05$).

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762 Table 4. Number of neural and haemal arches, vertebrae and parapophysis (mean ± s.e.)
763 undergoing ossification in cod larvae fed three microdiets (PL3, PL1, NL1). The larvae were
764 stained in Alizarin Red. Different letters indicate significant differences between the diets at
765 that day ($p < 0.05$).

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Dph	Diet	SL (mm)	No. larvae	Neural arches	Vertebrae	Haemal arches	Parapophysis
21	PL3	7.1 ± 0.2	7	0.0 ± 0.0[b]	0	0	0
	PL1	6.9 ± 0.04	6	0.2 ± 0.2[a, b]	0	0	0
	NL1	7.6 ± 0.08	7	0.7 ± 0.3[a]	0	0	0
25	PL3	7.9 ± 0.05	16	3.8 ± 1.0	2.5 ± 1.1	0	0
	PL1	8.2 ± 0.03	16	2.8 ± 0.7	1.6 ± 0.7	0	0
	NL1	7.9 ± 0.1	15	2.5 ± 1.0	1.1 ± 0.8	0	0
31	PL3	8.5 ± 0.06	12	14.8 ± 3.9[a]	12.8 ± 3.3[a]	4.7 ± 3.5	0
	PL1	8.4 ± 0.06	11	10.1 ± 3.7[a, b]	8.9 ± 3.0[a, b]	2.2 ± 1.7	0
	NL1	8.4 ± 0.08	13	6.2 ± 2.6[b]	4.3 ± 1.6[b]	1.7 ± 1.7	0
35	PL3	8.7 ± 0.05	17	17.8 ± 3.4	15.0 ± 3.6	7.8 ± 2.0	1.9 ± 0.3
	PL1	8.9 ± 0.02	22	22.5 ± 2.9	19.6 ± 3.6	8.2 ± 2.0	1.1 ± 0.2
	NL1	9.5 ± 0.08	15	21.7 ± 4.2	19.4 ± 4.3	8.7 ± 2.5	1.3 ± 0.3
45	PL3	13.3 ± 0.09	18	48.2 ± 1.0	43.3 ± 2.0	30.1 ± 0.8	7.8 ± 0.7
	PL1	12.5 ± 0.07	17	48.1 ± 1.3	44.8 ± 2.2	31.4 ± 1.1	8.4 ± 0.5
	NL1	13.3 ± 0.01	18	46.2 ± 1.5	43.2 ± 1.5	27.3 ± 1.6	8.5 ± 0.6

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769

770 Table 5. Cod larval mean number of fin rays on 45 dph (mean values ± s.e., n= 18 – 22 larvae
771 per treatment), after feeding experimental microdiets from 17 dph. Different letters indicate
772 significant differences between the treatments. Correlation between ray numbers and larval
773 size, r2: Pearson correlation, $p < 0.01$.

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Character	PL3	PL1	NL1	Correlation
1st dorsal fin	10.3 ± 0.3 [a]	8.6 ± 0.5 [a, b]	7.0 ± 0.8 [b]	r^2= 0.503
2nd dorsal fin	16.6 ± 0.5 [a]	13.8 ± 0.7 [b]	12.2 ± 0.7 [b]	r^2= 0.436
3rd dorsal fin	16.2 ± 0.3 [a]	14.0 ± 0.5 [b]	12.6 ± 0.8 [b]	r^2= 0.446
1st anal fin	18.2 ± 0.7 [a]	14.6 ± 0.7 [b]	15.7 ± 0.9 [a, b]	r^2= 0.541
2nd anal fin	16.1 ± 0.4 [a]	14.0 ± 0.4 [b]	12.9 ± 0.6 [b]	r^2= 0.356
Caudal fin	40.9 ± 0.7 [a]	36.5 ± 1.2 [b]	34.1 ± 0.9 [b]	r^2= 0.530

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Legends to the figures

Figure 1. Cod larval dry weight during the experiment (mean ± standard error of the means (s.e.), each point represents 45-67 larvae). Points with different superscript denote significant differences between dietary treatments ($p < 0.05$). “Co-feeding” represents the weaning period where larvae were fed enriched rotifers and increasing ratios of the experimental diets. The larvae were fed only experimental diets from 24 dph.

Figure 2: Cod larval content (mean values + s.e., n = 3) of docosahexaenoic acid (DHA), eicosapentaenoic acid (EPA), and arachidonic acid (ARA) at the start (17 dph) and during the microdiet feeding experiment. Points with different superscript mean that values between larval contents are significantly different on the specific sampling day ($p < 0.05$).

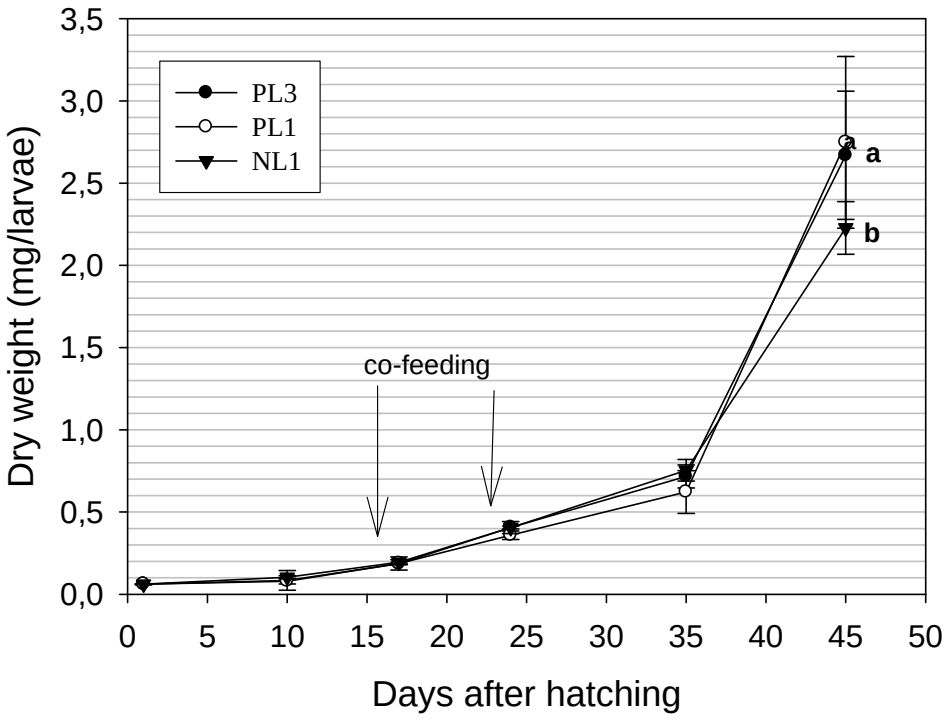
Figure 3: Skeletal development in cod larvae at the end of the experiment (45 dph, diet PL3). a) Larva stained with Alcian Blue and Alizarin Red, demonstrating formation of all fin rays. b) Larva with ossificiation of all elements of the vertebral column, stained with Alizarin Red. The calibration bar equals 1 mm.

Figure 4: Number of neural arches (a), vertebrae (b), and haemal arches (c) undergoing ossification in cod larvae fed the experimental microdiets, in relation to diets and larval size. Each point represents one larva (21, 25, 31, 35 and 45 dph), and the best fit curve found was the Gompertz growth model. PL3: Slashed lines, PL1: solid lines, NL1: dotted lines.

Figure 5: Mean number of neural arches (a), vertebrae (b), and haemal arches (c) undergoing ossification, in relation to diets and larval size inetrvals. Delayed ossification of haemal arches was found in NL1-larvae of 10.5-11.49 mm SL. Similar tendencies could e found for all ossifying elements of the vertebral column between SL of 8.5-11.5 mm, but individual variation within treatments was high.

Figure 6. Skeletal deformities in cod larvae from the experiment. a) Percentage of cod larvae with deformities of the vertebral column from the different dietary treatments (n = 15 x 3 for each column). (b) Percentage of each vertebral deformity type found from each treatment on 35 and 45 dph. No differences were found between the treatments, giving a total of 11% spinal deformities in the larvae on 45 dph.

Figure 7: Skeletal deformities were found in 11% of the cod larvae at the end of the experiment (45 dph), with shortened vertebrae and kyphosis accounting for 81% of the spinal malformations. a) Kyphosis in larva fed NL1-diet. The first eight vertebral bodies and their respective neural arches were deformed. The centre of the deformation is located on the 4th vertebral body, resulting in an A-shape. b) A 45 dph larva (PL1) with fused, shortened and dislocated vertebral bodies in the anterior part of the spine and deformed neural arches of the involved vertebral bodies. The larvae were stained with Alizarin Red, and the calibration bars equals 1 mm.



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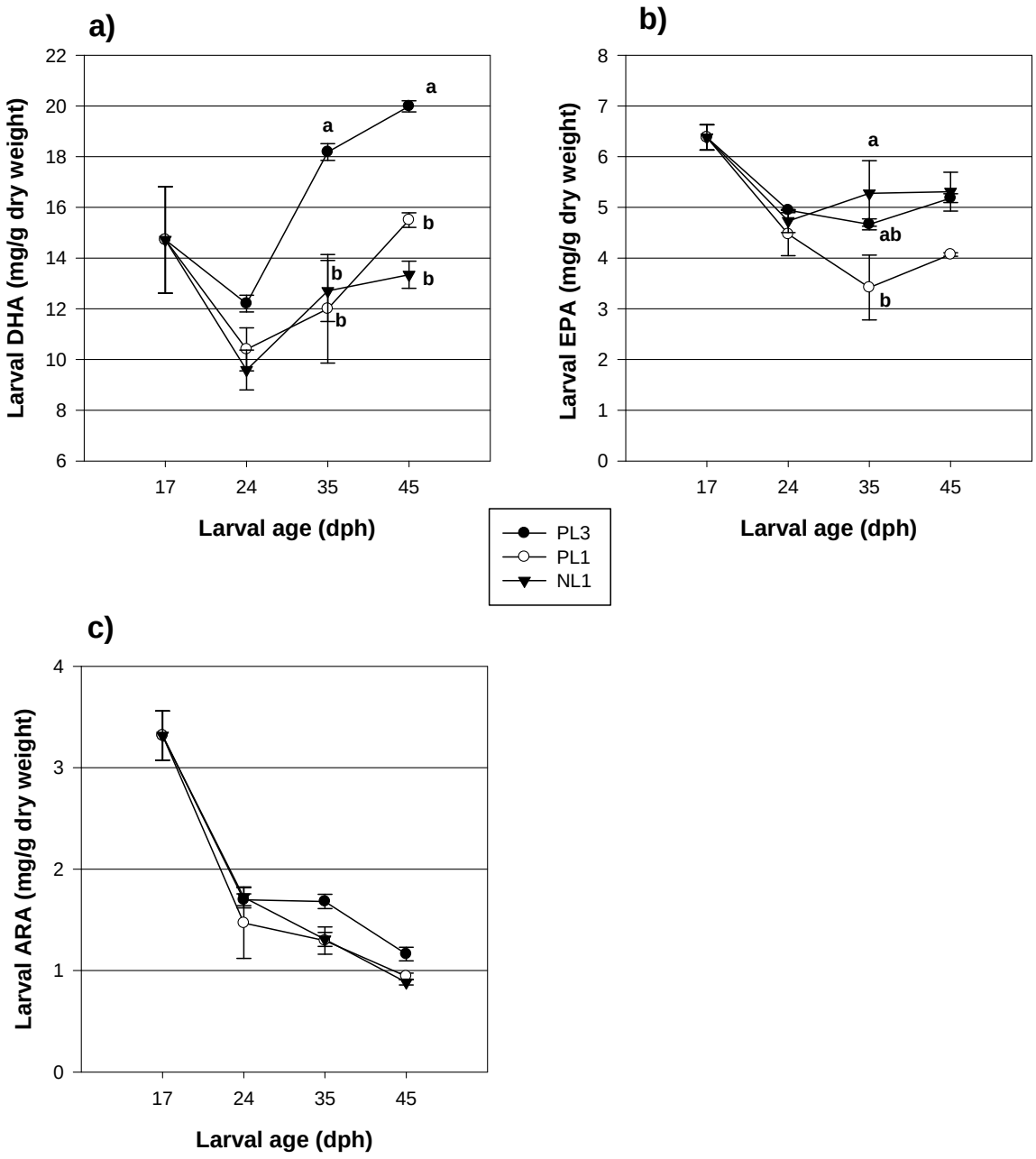

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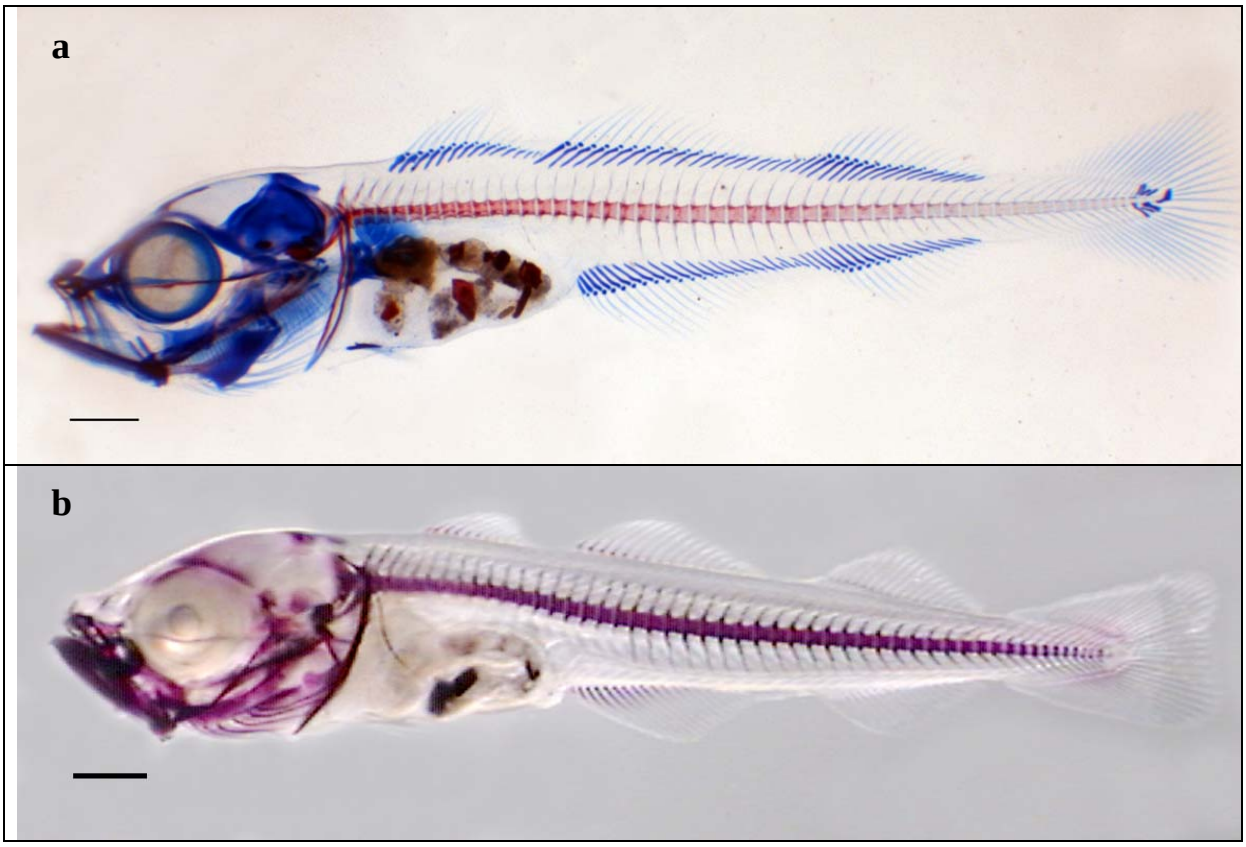

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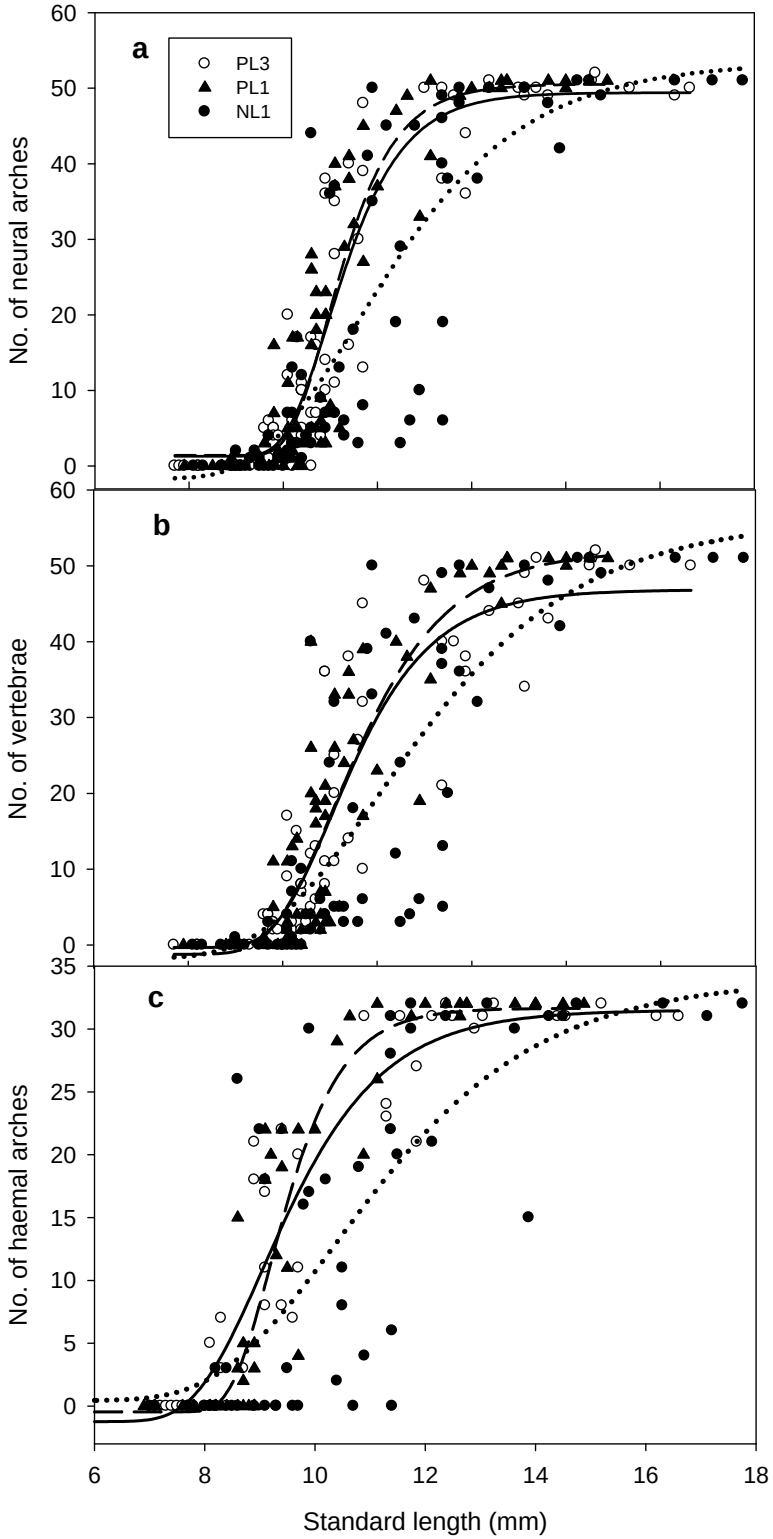

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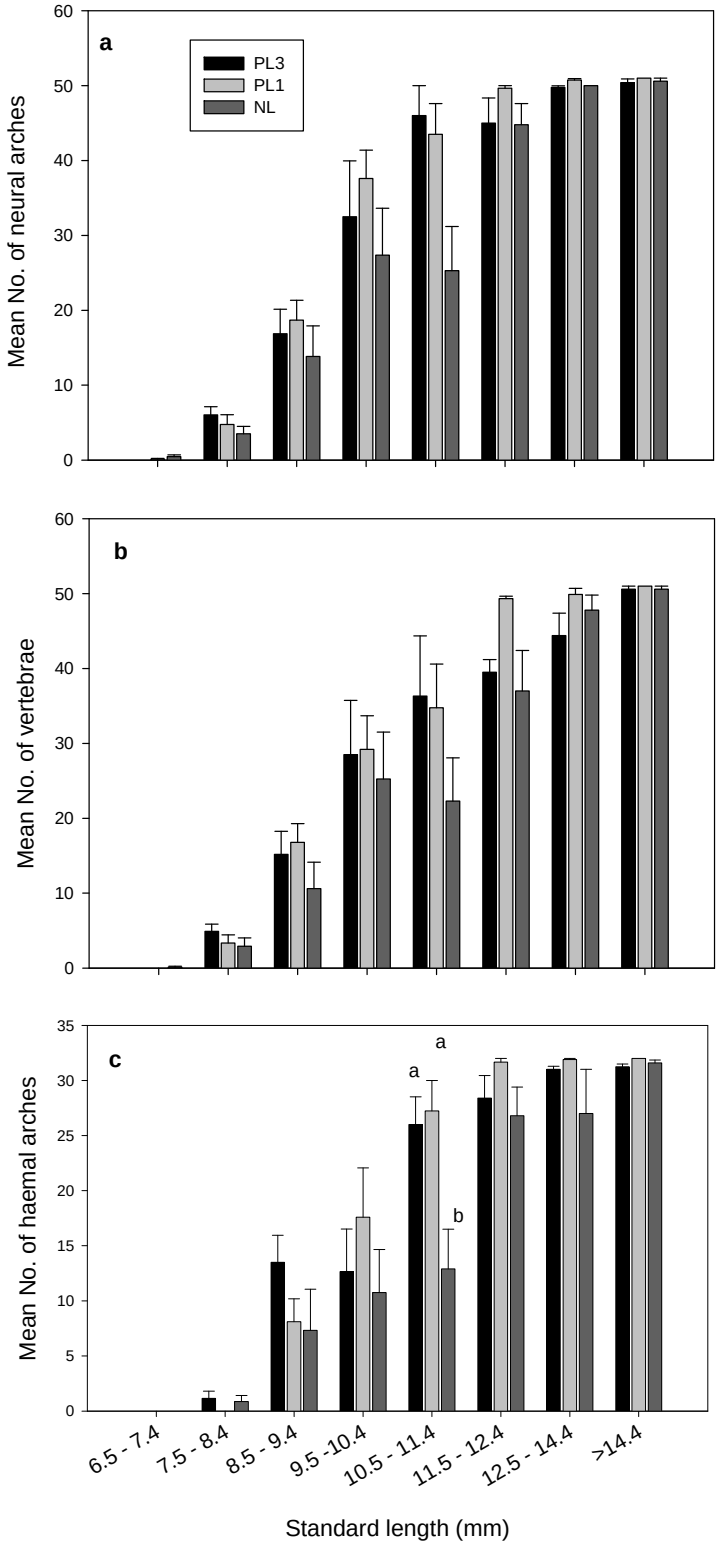

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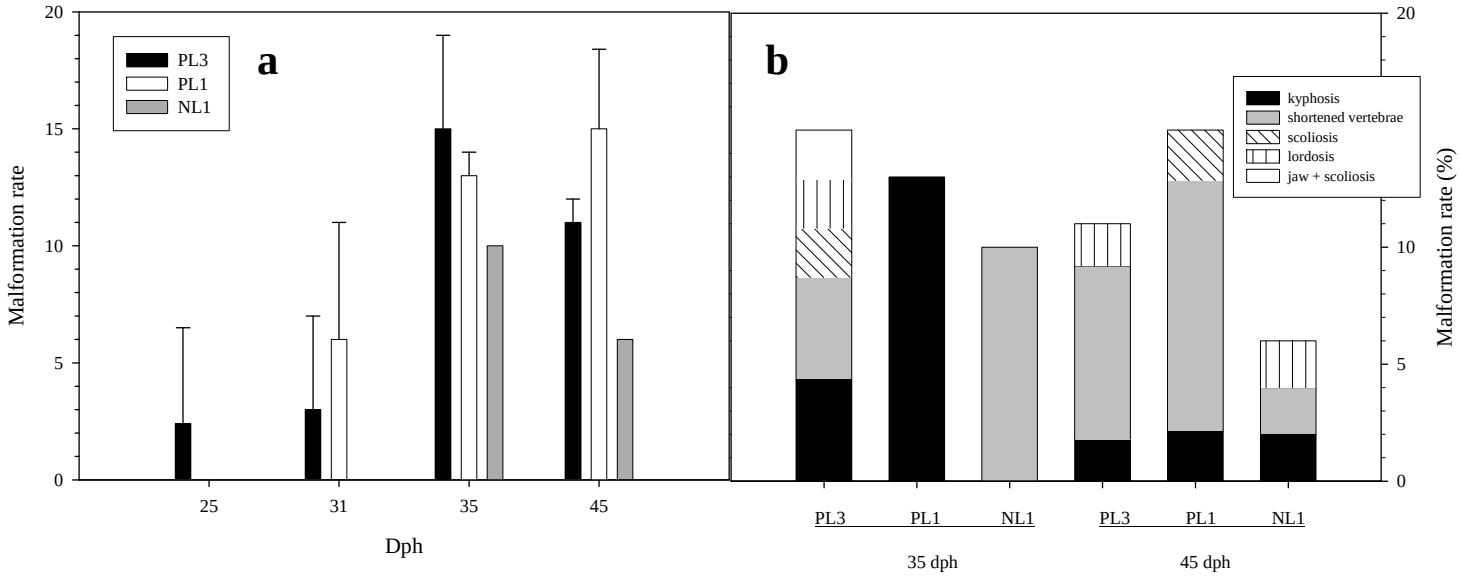

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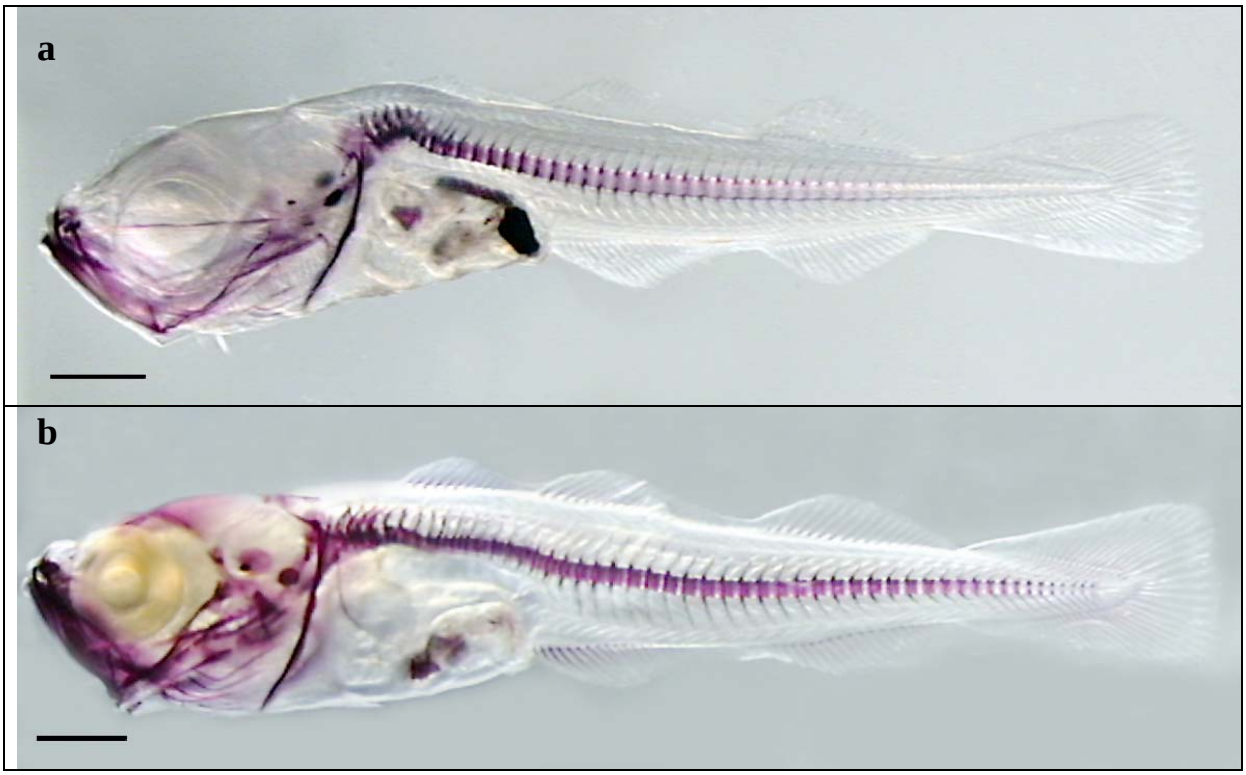

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