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Ossification of Atlantic cod (*Gadus morhua*) – developmental stages revisited

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Abstract

In studies of marine larvae, it is common to use days post-hatch as a developmental reference point. We show that age is a poor measure of morphological and physiological development in Atlantic cod. Therefore, we propose a set of five developmental stages of Atlantic cod from start-feeding until the juvenile stage, based on cranial ossification as previously done in Atlantic halibut. Cod follows a sequence of cranial ossification that is to a large extent preserved in most fish species examined. These stages are therefore tools to standardize sampling and to reduce growth dependent variation in the analysis of larvae during development. We show that several developmental stages are present in the same rearing unit at a given time. We also demonstrate that nutrition during early development is a vital foundation for robust skeletal development. Cod larvae supplied with copepods instead of rotifers followed by *Artemia,* develop less skeletal deformities at 10 cm standard length, despite given the same formulated feed from 1.8 cm standard length and onwards.

Introduction

In teleosts, there are commonly three developmental trajectories to become juveniles: indirect, intermediate, and direct development. Atlantic cod (*Gadus morhua* L.) is a species with indirect development, which means that the hatched larvae does not look like its adult form. When cod hatches from the egg it has a short period as a free living embryo (yolk sac larvae), before first exogenous feeding that marks the start of its larval period (Balon, 1999; Sars, 1879). The transition from the larval to the juvenile form involves complete organ "reprogramming" and further development, referred to as metamorphosis. The larval life is also remarkable in other ways, the sheer increase in weight itself is tremendous: in Atlantic cod there is a mass-increase of 2000 times during the first 50 days after first exogenous feeding (Finn et al., 2002).

There is a range of specific events that take place during metamorphosis: Larval muscle consists of thin fibres with few myofibrils whereas the adult type fibres are thick and rich in myofibrils (Yamano et al., 1991). The transformation also involves changes from larval isoforms of troponin-T and myosin light chains to the adult isoform (Inui et al., 1995; Yamano et al., 1991). The erythrocyte population changes from a larval form, consisting of large round cells to the adult smaller and elliptical form (Inui et al., 1995), with appearance of haemoglobin in late metamorphosis (personal observations). The gastric tract can roughly be divided into three regions at first exogenous feeding; fore-gut, mid-gut, and hind-gut. The pancreatic enzymes and bile are present at first-feeding in Atlantic halibut as well as cod (Gawlicka et al., 2000; Kortner et al., 2011; Sæle et al., 2011; 2010). Cones are present in the eye at hatching, but rods appear at metamorphosis (Valen, 2016). The olfactory epithelium is functional in the larval stages but the olfactory pit with its lamellae does not develop until metamorphosis in Barfin flounder (Yamamoto et al., 2004).

In studies of marine fish larvae, it is common to describe the larvae at a given age in days post-hatch (dph) or days post-fertilisation (dpf). We have previously shown that age is a poor measure of morphological and physiological development of halibut larvae (Sæle and Pittman, 2010; Sæle et al., 2004). Therefore, we propose a set of

defined developmental stages of Atlantic cod from start feeding until the juvenile stage, based on cranial ossification as a tool to standardize sampling and reduce the variation in the analysis of cod larvae. It has previously been shown that several developmental stages are present in the same rearing unit at a given time (Sæle and Pittman, 2010).

Skeletal deformities during the larvae stage has been a major challenge in the aquaculture industry, affecting fish welfare and product quality. Inadequate nutrition is proposed to be one of the major factors causing these skeletal deformities in intensively reared fish (Cahu et al., 2003; Hamre et al., 2013; Imsland et al., 2006; Lewis-McCrea and Lall, 2007). Zooplankton and copepod nauplii constitutes the main natural diet for first feeding marine fish in the wild, including Atlantic cod (Last, 1978; Wiborg, 1948). Analysis of the nutritional composition of natural zooplankton and of the rotifer/*Artemia* given to cod during intensive rearing, reveals large differences between the two (Hamre et al., 2013; van der Meeren et al., 2008). Fatty acid composition, vitamin and mineral composition and protein content differ. When cod larvae start to prey on copepod nauplii in the wild, this coincides with the initiation of skeletal ossification. At this point the skeleton has yet to ossify, (Hunt von Herbing et al., 1996a). As ossification takes place after exogenous food intake has started, nutrition obviously has a potential effect on ossification of the cod larvae. Previous studies have shown that the Atlantic cod larvae fed natural zooplankton grew faster than fish fed the intensive diet of rotifers and *Artemia* (Busch et al., 2010; Evjemo et al., 2003). Other studies have demonstrated that feeding zooplankton decreased the proportion of deformities in cod larvae compared to larvae fed the intensive diet of rotifers and *Artemia* (Fjelldal et al., 2009b; Imsland et al., 2006).

The first experiment of this study was used to establish developmental stages. These stages were used to define the sampling points of the second experiment that was designed to investigate the impact of nutrition on the initial ossification and skeletal deformities. We have therefore compared bone development in late larval stages and further in juvenile cod of comparable sized fish groups in fish fed either natural

zooplankton or enriched rotifers. A new and improved enrichment strategy was used for the rotifers (Karlsen et al., 2015).

Materials and Methods

Sampling and analyses

Experiments and sampling were carried out at the Institite of Marine Research at Austevoll and followed the Norwegian animal welfare act guidelines, in accordance with the Animal Welfare Act of 20th December 1974, amended 19th June, 2009. The facility has a general permission to conduct experiments involving all developmental stages of fish (code 93) provided by the Norwegian Animal Research Authority (FDU, www.fdu.no).

Experiment 1

To characterize and specify the larval ontogeny into developmental stages ten cod larvae from each of triplicate tanks were collected on 4, 7, 13, 19, 28, 34, 41, 48, 59, and 66 dph from the following experiment: 10 dl (ca. 50 000 eggs) fertilised naturally spawned eggs were incubated and hatched in 70 litre tanks, with a similar temperature to the brood stock, 6°C. The majority of the eggs were hatched after 15d, with a hatching rate of more than 80 %. 16 250 larvae were then transferred to the first feeding tanks (400 litres, with central aeration). Water temperature was gradually raised from 6 to 12 °C over 6 days. The larvae were first fed rotifers from 4 dph, then received *Artemia* from 33 dph and were gradually weaned onto dry feed from 36 dph.

Experiment 2

To evaluate the dietary impact on ossification of the standard rotifer/*Artemia* diet used in intensive production of cod versus a “natural” diet of various stages of copepods, cod larvae from a large-scale nutrition study were analysed. The detailed description of this experiment is given in Karlsen et al., (2015) where it is referred to as

Experiment 1. Figure 2 in Karlsen et al., (2015) describes the experimental setup and feeding regimes in this nutrition study. Briefly, one group of cod larvae were fed a standard live diet, first rotifers (4-35 dph), then *Artemia* (32-63 dph), and finally weaning was initiated and completed on 55 and 64 dph, respectively. Based on the developmental stages described from the first experiment, sampling of this experiment was executed when the fish larvae had reached its developmental stage. This strategy resulted in sampling at 4, 11, 22, 31, 54, 71, and 85 dph in the rotifer/*Artemia* group. Ten larvae were collected from each tank. The other group was fed only copepods collected from a pond (van der Meeren et al., 2014), starting with nauplii at 4 dph and including successively larger prey (copepodites) as larval size and age increased, with start and completion of weaning on 37 and 45 dph, respectively. From this treatment, ten cod larvae were sampled from each triplicate tank on 4, 11, 22, 29, 37, 53, and 74 dph, which was different from the rotifer/*Artemia* treatment due to divergence in growth between the two treatments (Karlsen et al., 2015).

Photographs were taken of anesthetized (overdose of metacain, Argent Laboratories) larvae prior to fixation in 4 % formalin buffered in PBS. Larvae used for gene expression analyses were sampled at the same days but in larger numbers (see Table 1).

Clearing and staining for bone

Staining with Alizarin red S was performed according to Sæle et al., (2003). Euthanized fish larvae were fixed in 4% PBS (pH 7.2) buffered formalin overnight. Staining with Alizarin red S was performed according to the following procedure: Three hours immersion in a 0.1 M solution of NaOH, containing 0.18% alizarin red ($C_{14}H_7O_7SNa$) (Merck, Darmstadt, Germany), followed by washing four times for 30 min in distilled water. Thereafter the larvae were dehydrated in 70% ethanol overnight, followed by dehydration in 90% ethanol for 1 h. Larvae were preserved and cleared in 87% glycerol (Merck).

Alizarin red binds to calcium and is visualised as red/orange in calcified areas (Potthoff, 1984). Branchiostegal rays were not considered due to their fragility, which could represent a potential source of error.

All stained larvae from experiment 1 were examined for progress in cranial ossification. The results were scored from 1 to 3 to illustrate trends in the appearance and fusion of bones. The score distinguished between the presence of mineralization in a bone (1), the establishment of a distinct bone shape (2), and finally, when the bone had the shape like in adults (3). When treatment differences in ossification occurred, the larvae were re-examined to determine the bones involved. All scoring was done by the same person. The ossification scores were then summed up for each individual. Stage classification was based on increases in the score. An overview over bones included is given in Fig. 1. The stained larvae were also investigated for skeletal deformities.

Images

Imaging was done on a AZ100 multizoom makroscope (Nikon, Japan) with a AZ Plan Apo 1x objective (Nikon, Japan) with DS Fi1 (Nikon, Japan) for bright field images. Confocal scan was done with the $C2^{+}$ confocal scanner (Nikon, Japan). The confocal scanner settings were; first filter cube: 447/60, second filter cube: 525/50 561 LP, emission wavelength: 785 nm, laser power: 3.4 and pinhole size: 30 μm^2.

Radiography

Juvenile cod from experiment 2 were X-rayed at the Nofima radiography laboratory in Sunndal, Norway. The fish were kept frozen during storage and through X-ray analysis. The material consisted of 300 cod juveniles, 50 fish from each of the six tanks, of which 3 tanks were from the copepod fed group and 3 tanks from the rotifer/*Artemia* fed group. There was a size difference between the two treatment groups, with fish from the copepod fed group measuring approximately 100-120 mm, and rotifer/*Artemia* group measuring 70-90 mm.

The radiographic imaging was performed on frozen fish using a semi-digital computed radiography system (Fuji Medical AS, Oslo, Norway). Images were recorded on FCR Imaging Plates (Fujifilm, Tokyo, Japan) coated with photo-reactive phosphorus, and read in a FCR Profect reader (Fujifilm, Tokyo, Japan). Image enhancement was performed automatically by FCR console software, using fish size specific settings to obtain optimal image quality. An IMS Giotto mammography X-ray (Giotto, Pontecchio Marconi, BO, Italy) source was used, with exposure at 22 kV and 100 mAs, in combination with image plates with double-sided coating, resulting in an image resolution of 20 pixels mm^{-1}. The images were visually examined for any distinct skeletal pathology, and compared to relevant descriptions of skeletal morphology and pathology in this species (Fjelldal et al., 2009b).

As an indicator for potential cranial lordosis (Stargazing, bent neck), the angle between head and spine was measured. The angle was defined by drawing two lines, the first from the front end of the palate bone to the centre of the first vertebra, and the second from the centre of the first vertebra to the centre of vertebra no. 6. ImageJ software was used to find the angle between the two lines (ImageJ 1.47v, National Institutes of Health, USA, www.imagej.nih.gov/ij).

The size difference between the two treatment groups resulted in a difference in image quality due to resolution limitations, which to some extent affected evaluation. In particular, the most caudal vertebrae were less clear in images from the smaller fish, i.e. the rotifer/*Artemia* group. Consequently, observations of pathology in the caudalmost vertebrae were excluded from the evaluation for both groups, as a valid comparison could not be made. For the rest of the vertebral column, i.e. excluding the most caudal vertebrae, image quality was consistently good enough to evaluate vertebral morphology in both groups.

RNA extraction and qPCR

Frozen larvae were homogenized on Quiazol (Qiagen) using a Precellys 24 (Bertin

Technologies). Total RNA was extracted from the whole fish on a Bio Robot EZ1 using the EZ1 RNA Universal Tissue Kit with the RNase-free DNase Set (Qiagen), according to the manufacturer's instructions. The quantification and purity of RNA was assessed with the NanoDrop^{W} ND-1000 UV – Vis Spectrophotometer (NanoDrop Technologies). For all total RNA samples, the optical density ratio at 260/280 nm ranged between 1.93 and 2.19. Integrity of RNA was controlled in twelve randomly chosen samples out of the thirty-six using the Agilent 2100 Bioanalyzer and the RNA 6000 Nano LabChip^{W} kit (Agilent Technologies). RNA integrity numbers (Imbeaud, 2005; Mueller et al., 2000) were between 8.2 and 9.7. Aliquots of RNA samples were saved in case qPCR outliers were spotted and RNA needed integrity analyses, however, no outliers appeared in this study.

RT reactions and qPCR were run according to Sæle et al., (2013). Genes coding for proteins involved in mineral and bone and cartilage deposition (matrix gla protein (*mgp*), bone gla protein 1 (*bglap1*), bone gla protein 2 (*bglap2*), transcription factor sp7 (*sp7*) and periostin (*postn*), collagen 1a2 (*col1a2*) and collagen 10a1 (*col10a1*)) and bone resorption (cathepsin K (*ctsk*), Receptor activator of nuclear factor kappa-B ligand (*rankl*) and osteoprotegerin (*opg*) were selected as target genes. Results were calculated as the arithmetic mean using ubiquitin (ubi) and beta-actin as reference genes based on the study of Sæle et al., (2009). The genes were normalized using the geNorm visual basic for applications applet for Microsoft as previously described by Vandesompele et al., (2002). Primer sequences, product size and PCR efficiency for each quantitative PCR assay are given together with the corresponding accession number in Table 2.

Calculations and statistics

To find the best curve fitting and calculate the coefficient of determination (R^2) for age or length versus stage or sum of bone score, GraphPad Prism version 6.0 for Mac OS X, GraphPad Software, La Jolla California USA, was used. Differential expression following qPCR was analysed using the Statisitca 12 software package (StatSoft, Tulsa, OK, USA) and main effect ANOVA with Bonferoni post hoc test. Differences and effects were considered significant at $p < 0.05$ for all tests.

Results

In the staging experiment, cranial ossification in the cod larvae started with structures in the jaw, namely the *maxillary* of the upper jaw and the *dentary* of the lower part. These structures ossified before the larvae reached 6 mm SL. The largest bone of the branchial arches, the *ceratobranchial* also started to mineralize at this size. The next elements to ossify were the *premaxillary*, *articular* and the *angular* of the jaw, as well as the *symplectic*, *ceratohyal* and *hypohyal* of the hyoid arch. The first structures of the neurocranium, the *parasphenoid* and the *exo-* and *basi-occipitals* were starting to mineralize at the size of 6 to 7 mm as well. The successive ossification of the lower jaw next is the *quadrate*, followed by *ectopterygoid* and eventually the *mesopterygoid*. In the hyoid arch the hyomandibular, urohyal started to ossify at the same time as the structures of the opercular; the *praeopercular*, *subopercular*, *interopercular* and the *opercular*.

Next to mineralize was the *nasal*, *frontals*, *parteial*, *vomer*, *pterotic* followed by the *basioccipital* of the neurocranium and the *hypobranchials* of the branchial arches. The last of the bones in this study to mineralize was *palatine* in the suspensorium, the *epihyal* followed by *interhyal* in the hyoid arch. In the neurocranium the ethmoid bone group in the snout region, *pterosphenoid*, the otic bone group (*sphen-*, *epi-* and *opistoticum*), the *supraoccipital* of the occipital group and the *orbital* bones circumferencing the eyes were the next to ossify.
Based on the sequence and degree of mineralization of the cranial bones in the staging experiment, the five following stages are proposed (Table 3). The stages cover the time from when the larvae started eating until they obtained the characteristics of juvenile cod.

Stage 1: The cranium is characterized by initiation of ossification of the jaw, including the *maxillary* and *dentary*, and the *cleithrum* (data not shown) ossifies. Other characteristics for this stage: Yolk sac is absorbed, intestine is looped, and there is a

primordial fin. The notochord is thicker than the surrounding muscle layer and is the major structure of the body trunk (Fig. 2).

Stage 2: The cranium is characterized by the ossification of the jaw. *Premaxillary* and *maxillary* of the upper jaw and *articular*, *angular* and *dentary* of the lower jaw are now mineralized. *Ceratohyal* and *symplectic*, parts of the hyoid arch are starting to mineralize during this stage. In the neurocranium there is the *parasphenoid*, *exoccipital* and *basioccipital* starting to mineralize. In the brachial arches, ossification in four of the five *ceratobranchials* is now commencing. Other characteristics for this stage: the muscle layer surrounding the notochord is now thicker than the notochord (Fig. 2).

Stage 3: The bones of the jaw, in particular the maxillary bones, *dentary* and *articular* have attained a more recognizable shape. There is also mineralization in the *angular*, *quadrate* and late in the stage in the stage the *ectopterygoid* as well. In the neurocranium, both the *parasphenoid* and the *basisphenoid* as well as the *basioccipital* and *exoccipital* are attaining a mineralized form resembling the juvenile shape. In some individuals, the ossification of opercular structures may be observed. Other characteristics for this stage: the muscles are the most abundant tissue composing the body trunk. The dorsal primordial fin fold is now starting to differentiate into the three dorsal fins. In some individuals, red blood may be observed where they are most concentrated, in the heart and the gill structures. Flexion starts at the end of this stage (Fig. 2).

Stage 4: Most cranial structures have some level of mineralization. The bones of the jaw attain a more adult-like shape and the *palatinum* is now mineralized. In the hyoid arch the *hyomandibular* is present, and the *epihyal* and *urohyal* appear. The opercular bones attain an adult-like shape. In the neurocranium the *nasal*, *frontals*, *parteial*, *pterotic* appear and the *supraoccipital* start to ossify. In stage 4 all five *ceratobranchials* are mineralized. Also the *epibranchials* and *hypobranchials* mineralize during this stage. Other characteristics for this stage: differentiation of the

fin fold is finalized and fin rays are clearly visible. Flexion is completed at the beginning of this stage. (Fig. 2).

Stage 5: This stage is best characterized by the "closing" of the neurocranium, by the expanding ossification of the otic group, in particular the *supraoccipital*, *parietale* and frontals. The profile of the snout is changing from being curved in the ventral direction to a more straight snout. This is due to the development of the *nasal* and the ethmoid group. New to this stage are the orbital bones surrounding the eyes. In the hyoid arch the *epihyal* may now be identified by its shape and the *interhyal* is also present (Fig. 2).

Juvenile (stage 6): Nearly all bones are now present, and particularly the advanced ossification of the *nasal*, the ethmoid group together with the *frontal* processes change the profile of the snout to the characteristic adult shape. Other characteristics for this stage: the transition from the final larval stage to a juvenile cod is characterized by the complete, adult type pigmentation. Even though pigment cells are distributed over the whole body from stage 4, it is not until the juvenile stage the cod has its characteristic dark back and white belly pigmentation (Fig. 2).

The relationship between the proposed developmental stages and standard length (SL) as well as stages related to age was also investigated. The relationship between the sum of ossification score and age was $R^2 = 0.87$, and the sum of ossification score and SL was $R^2 = 0.98$. When grouping the fish larvae in the developmental stages the relationship was $R^2 = 0.87$ and $R^2 = 0.90$ accordingly (Fig. 3).

Ossification of vertebrae

Ossification of the vertebral column first appears during stage 2 (Fig. 4B), starting with the most anterior vertebrae. Ossification then gradually proceeds in the caudal direction during stage 3 (Fig. 4C), and most of the vertebrae are mineralized at stage 4 (Fig. 4D), even though the bones in the tail region are still poorly mineralized. The whole axial skeleton is mineralized during stage 5 (Fig. 4E).

Larval cod from the feeding experiment at stage 5 and the juvenile stage, stained with alizarin red for visualizing bone mineralization, were examined for vertebral abnormalities. No abnormalities were observed in the 48 copepod fed cod that were analysed. Out of the 48 rotifer/*Artemia* fed cod examined, only two showed signs of vertebrae fusion in the neck region. In contrast, when the fish had grown into the size range of 7 to 12 cm SL, 5% of the copepod fed fish and 33% of the rotifer/*Artemia* fed fish showed signs of vertebrae abnormalities as examined with x-ray (Fig. 5B).

Vertebral lesions identified with x-ray were mainly of the ankylosis (fusion) type, observed both as complete ankyloses, where vertebral centra were completely fused (Fjelldal et al., 2009b), or as developing ankylosis, in which vertebral centra were still separate at the time of examination, but which should be considered as early stages of a fusion process (Witten et al., 2006). Ankylosis in various stages was the dominant type of pathology in both treatment groups, representing 4% and 32% of fish examined in the copepod and rotifer/*Artemia* fed groups, respectively. The pattern of lesions differed between treatments. A particular high incidence of fusions was observed in the neck region, i.e. affecting the anterior vertebrae, representing 1% of fish in the copepod fed groups and 24% of fish in the rotifer/*Artemia* fed groups. Remaining lesions were distributed along the spinal column. Severity of lesions, counted as number of deviant vertebrae per affected fish, displayed no significant difference between fish groups (Fig. 5). In addition to fusions, one fish in the copepod fed group had a vertebral lesions described as compressed vertebra (Fjelldal et al., 2009b). Minor deviations in the angle of the lower jaw were recorded in two fish, one from each treatment group. No haemal lordosis was observed.

Measurements of angles between the head and spine (cranial lordosis) did not differ between treatment groups, with an average value of 187° in both groups, which is considered normal. There was, however, variation in neck angle within each of the groups. Recorded values ranged from 171 to 196°. The standard deviation as well as the range was the same for both fish groups.

Additional observations made on the largest fish, the copepod fed group, indicated a high incidence of minor lesions in the caudal most vertebrae. The lesions were typically complete fusions between two vertebrae. This type of lesion was identified in 39 of 150 fish in the copepod fed groups. Due to limitations in image resolution related to smaller fish size, corresponding data from the rotifer/*Artemia* fed fish could not be recorded consistently. Thus, these data were omitted from further evaluation, and are not reported beyond this note.

qPCR

Separate gene expression analysis was conducted for stage 1-3 (when the fish were fed either rotifers or copepods), for stage 3 (*Artemia* or copepods) and for stage 4-6 (Fig. 6). For stages 1-3, *col1a2*, *col10a1*, *postn* and sp7 (osx) was down-regulated rankl was up-regulated in rotifer fed fish compared to copepod fed fish (Fig. 6). None of the other investigated non-collagenous proteins like (*bglap1*, *bglap2*, *mgp* or *ctsk* were differentially expressed between the groups (Two–way ANOVA, $p<0.05$) in stages 1-3. No differences in expression for any of the examined genes were detected at stages 3-6 (Two way ANOVA $p<0.05$, Sidak's multiple comparisons test).

Discussion

Staging of Atlantic cod larvae

The sequence of cranial ossification in Atlantic cod follow the same sequence as the more primitive fish such as the Bowfin (*Amia calva*) (Schoch, 2006), golden Nile catfish (*Chrysichthys auratus*) (Vandewalle et al., 1995) zebrafish (*Danio rerio)*, (Mabee et al., 2000) brook trout (*Salvelinus fontinalis*) (Steingraeber and Gingerich, 1991) as well as other gadoid fishes such as walleye pollock (*Theragra chalcogramma*) (Brown et al., 2001). Starting with bones associated with feeding and then bones related to respiration, it is plausible that this sequence is driven by development of functionality in the different structures (Adriaens and Verraes, 1997; Mabee and Trendler, 1996; Sæle et al., 2004). Emphasizing the success of such a developmental pattern, this sequence is also found in more advanced species such as Japanese rice fish (*Oryzias latipes*) (Mabee et al., 2000), Siameese fighting fish (*Betta splendens*) (Mabee and Trendler, 1996), sea bream (*Sparus aurata*) (Faustino and Power, 2001), and flatfishes (Sæle et al., 2004). In fact, the sequence of the craniofacial ontogeny is conserved not only in fish, but also in tetrapods, from amphibians to mammals (Schoch, 2006). This indicates a conserved developmental feature and therefore advocates for the adoption of this as a basis for a general staging system.

Even though the stages described here are based on neurocranial ossification, a conserved developmental pattern, it is admittedly not a practical feature to use when sampling. Staining larvae for mineralized bone is a time-consuming task. As found earlier in Atlantic halibut (*Hippoglossus hippoglossus*) (Sæle et al., 2004), the easiest measurable proxy to describe the developmental stages based on osteo-cranial development is size. Length is therefore a useful proxy for description of ontogeny of cod larvae. Other studies have also established the higher correlation between size and development compared to age and development. Hutchinson and Hawkins, (2004) demonstrated that the metamorphosis of the European flounder (*Platichthys flesus*) was linked to size and not age. Rearing flounder larvae at different temperatures changed the growth rate and the age at which it metamorphosed significantly, but not

the size at which it metamorphoses.

Earlier efforts have been carried out to divide the larval period of Atlantic cod into stages (Hunt von Herbing et al., 1996b). Hunt von Herbing's stages are numerous, and the description of morphological development to identify the stages has proven difficult to implement in cod larvae. For example, she described cranial elements that are thicker and larger in one stage than in the previous stage. It has therefore unfortunately been difficult to compare our material and stages to that described by Hunt von Herbings. Herbing Hunt et al., (1996b) description of the morphological changes that define the stages of the developing intestine in cod is not comparable with our findings. The analysis of the developing intestine of cod in the present work corresponds with earlier findings (Kamisaka and Rønnestad, 2011; Pedersen and Falk-Petersen, 1992).

Effects of diet on bone development

In Atlantic salmon (*Salmo salar*) it has been demonstrated that suboptimal nutrition can cause a softer skeleton during development which can ultimately lead to pronounced skeletal abnormalities, such as compressed vertebrae and craniofacial abnormalities (Fjelldal et al., 2012). Vertebral compressions like those reported here in cod larvae might be a result of heterochronic bone growth/remodelling as a reaction to high workload (Fjelldal et al., 2012). However, there was no apparent differences in workload between the experimental groups in this trial, since all tanks had equal water exchange and water supply and draining systems resulting in similar water currents. We propose that the most likely explanation to the difference between experimental groups in vertebral compression is a nutritionally induced weakness in the vertebrae during early ossification. This compares with another study where similar deformity deviations were observed between cod of same genetic origin reared on copepods versus rotifers/*Artemia* in different rearing systems (Fjelldal et al., 2009b). It is likely that such differences in nutritional status may affect the expression of genes involved in bone growth and remodelling. However, no differences in expression of the non-collagenous matrix proteins, *bglap1, bglap2 and mgp*, were observed between the

groups at the onset of mineralization. Previously, it has been shown that both bone/cartilage ratio and mineralization patterning coincide with regulation of osteocalcin gene expression (Bensimon-Brito et al., 2012; Mazurais et al., 2008). Although we found a difference in col10a in stage 1-3, this expression cannot be attributed to bone only since analysis were performed on whole larvae. This gene is expressed in all types of connective tissue, not only the skeleton. Thus, the results indicate that the different diets in the present study did not affect the level of mineralization, as was one of the anticipated outcomes. Therefore, lowered mineralization did not appear to be the cause of vertebrate compression in fish that had been fed rotifers/*Artemia* during the larval stages. Accordingly, Witten et al., (2005) did not find any evidence of mineral deficiency in compressed vertebrates in salmon. However, Fjelldal et al., (2009a) found that both mineral content and strength of bone was reduced in fish belonging to the group that had the highest prevalence of deformities, indicating a relationship between deformities and bone mineral content.

Vertebral compression in adult salmon is believed to be caused by replacement of regular notochord tissue with ectopic cartilage through a change in osteoblast activity towards a chondroblast-like activity (Kvellestad et al., 2000; Witten et al., 2006; 2005). Although there were no differences in expression of non-collagenous matrix proteins between rotifer- and copepod-fed fish, one of the key genes regulating osteoblast maturation and activity, *osx*, was down-regulated in larvae fed rotifers compared to larvae fed copepods. We also observed that *rankl*, responsible for osteoclast activation, was up-regulated in the same samples. The balance between osteoclasts and osteoblasts drives bone development through a constant remodelling. The shift towards decreased osteoblast activity/maturation and increased osteoclast activity/maturation could result in an imbalance in bone remodelling and growth, which in turn could have an unfavourable outcome. However, it is not clear whether these differences in gene expression could result in the abnormalities observed in rotifer-fed fish in the present study. Nor did we observe any increased *ctsk* expression as could be expected if there were an increased osteoclast activity following *rankl* activation.

Moreover, no pronounced differences in deformities were observed between the two groups at stages 4 to 6. Mineralized and newly mineralized vertebrae anlagens appeared intact and normal. Lack of vertebrate abnormalities in the younger stages is in contrast to the increased prevalence of abnormalities observed in the rotifer/*Artemia* fed group sampled at a much later stage (around 10 cm total length). Ossification of vertebrae in cod is sequential in the caudal direction and the vertebrae of the neck are almost complete after the period when the larvae were fed rotifers and *Artemia*. This may explain why vertebrae in the neck were more damaged than in the caudal region in the rotifer/*Artemia* fed larvae compared to the larvae fed copepods. Furthermore, there seems to be a lag time between exposure to the different treatments and pronounced visual effects. The deformities which become apparent at later stages may have developed from subtle abnormalities much earlier, which could be difficult to detect using present staining techniques. Alternatively, the onset of vertebral fusion may have occurred after completion of vertebrate mineralization in response to some unidentified factor.

Vertebral deformities have been studied extensively in Atlantic salmon (reviewed by Fjelldal et al., 2012). Several critical stages, where bone deformity development commences, have been identified but the origin and causes of many deformities are often unclear. In Atlantic salmon reared at different temperatures through start feeding, the differences in vertebral deformities first became apparent at 15 g (Ytteborg et al., 2010). Fjelldal et al., (2007) showed that vertebral compression observed in fish 10 month after transfer to seawater was not apparent at the earlier parr stage, indicating a delayed display of abnormalities, as was found in the present study with cod.

We have here demonstrated that the sequence of cranial ossification in Atlantic cod is following the same pattern as in other examined teleost fish. The best proxy for developmental stages is size, age on the other hand reflects development poorly. By sampling fish larvae according to size and not age, one can more accurately relate different studies to one another. Nutrition during early development has consequences

for bone robustness later in life as demonstrated by the vertebrae deformities observed in older fish but not in young juvenile fish.

Contributors

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Conflict of interest

All authors declare no conflicts of interest.

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Table and figure texts

Table 1. Number of fish larvae and ages (days post hatching), sampled for gene expression analysis at stages 1 to 6.

Table 2. GenBank and Ensembl* Accession numbers for qPCR primer assays.

ubi = ubiquitin, bact = beta actin, ctsk = cathepsin k, postn = periostin, mgp = matrix gla protein, sp7 = transcription factor sp7/osterix, bglap1 = bone gla protein 1, bglap2 = bone gla protein 2, rankl = receptor activator of nuclear factor kappa-b ligand/ tumor necrosis factor (ligand) superfamily, member 11 and opg = osteoprotegerin/ tumor necrosis factor receptor superfamily, member 11b, col1a2 = collagen 1a2, col10a1 = collagen 10a1.

Table 3: Score table of cranial ossification in Atlantic cod. Each column shows data from individual larva analysed. Rows indicate score for individual bone. For bones of which there are several of the same type, the quantity of the specific type of bones are given in parenthesis.

Fig. 1: 3-D confocal cranial scan of a 26.6 mm SL cod stained with Alizarin red S. Bones used in Table 3 are named with the exception of the branchial bones (basi-, hypo-, cerato- and epibranchial) and the uro-, hypo-, cerato-, epi- and inter-hyal of the hyoid arch.

Fig. 2: Size range of stages 1 to 5. The yolk sac stage has been entitled 0 and the juvenile stage is entitled Stage 6 in the rest of the manuscript. Photos represent each of the developmental stages, and inserts represent the status of cranial ossification of the stages.

Fig. 3: Relationships between age or standard length and ossification score or stages. A: $\mathrm{SL(mm)} = 5.2398 * e^{0.0168\mathrm{Score}}$, $R^2 = 0.98$. B: $\mathrm{SL(mm)} = 3.052 * e^{0.3942\mathrm{Stages}}$, $R^2 = 0.90$. C: $\mathrm{Age(dph)} = -7.356 + 34.5009 * \log(\mathrm{Score})$, $R^2 = 0.87$. D: $\mathrm{Age(dph)} = 4.8124 + 80.7437 * \log(\mathrm{Stages})$, $R^2 = 0.87$

Fig. 4: Atlantic cod larvae claired and stained for bone (Alizarin red S), representing stages 1 to 5, from A to E. Note that mineralization of vertebrae does not occur before Stage 2 (see innsert in B). Nomeclature according to Cannon, D.Y., (1987)

Fig. 5: Radiography of cod juveniles. A) Normal fish (copepod fed) B) Fusion in neck vertebrae (rotifer/Artemia fed) C) Complete fusion and D) Incomplete fusion in trunk vertebrae (both rotifer/Artemia fed). Details of B-D in inserts. Lower panel:

Distribution of vertebral lesions in the spinal column of juvenile cod in rotifer/Artemia fed groups (red bars) and copepod fed groups (blue bars). X-axis show vertebrae number where 1 is the most anterior.

Fig. 6: Gene expressions presented as mean normalised expression (MNE) for *col1a2*: Collagen, TypeI, Alpha 2, *col10a2*: Collagen, TypeX, Alpha 2, *opg*: osteoprotegerin/tumor necrosis factor receptor superfamily member 11b, *rankl*: receptor activator of nuclear factor kappa-B ligand/ tumor necrosis factor (ligand) superfamily member 11, periostin, *osx*: osterix/transcription factor *sp7*. Rotifer fed larvae in red and copepod fed larvae in blue. Sampling point when the rotifer group were fed *Artemia* is marked: ●. Sampling points when the two experimental groups were fed formulated diets are marked: ●.

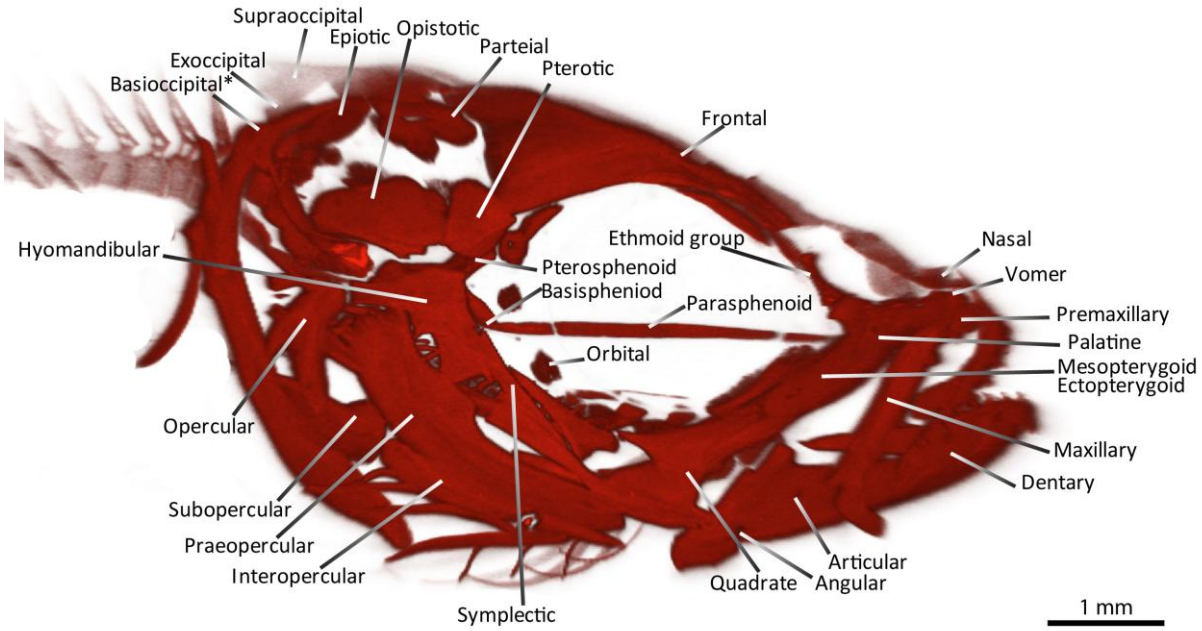

Figure 1

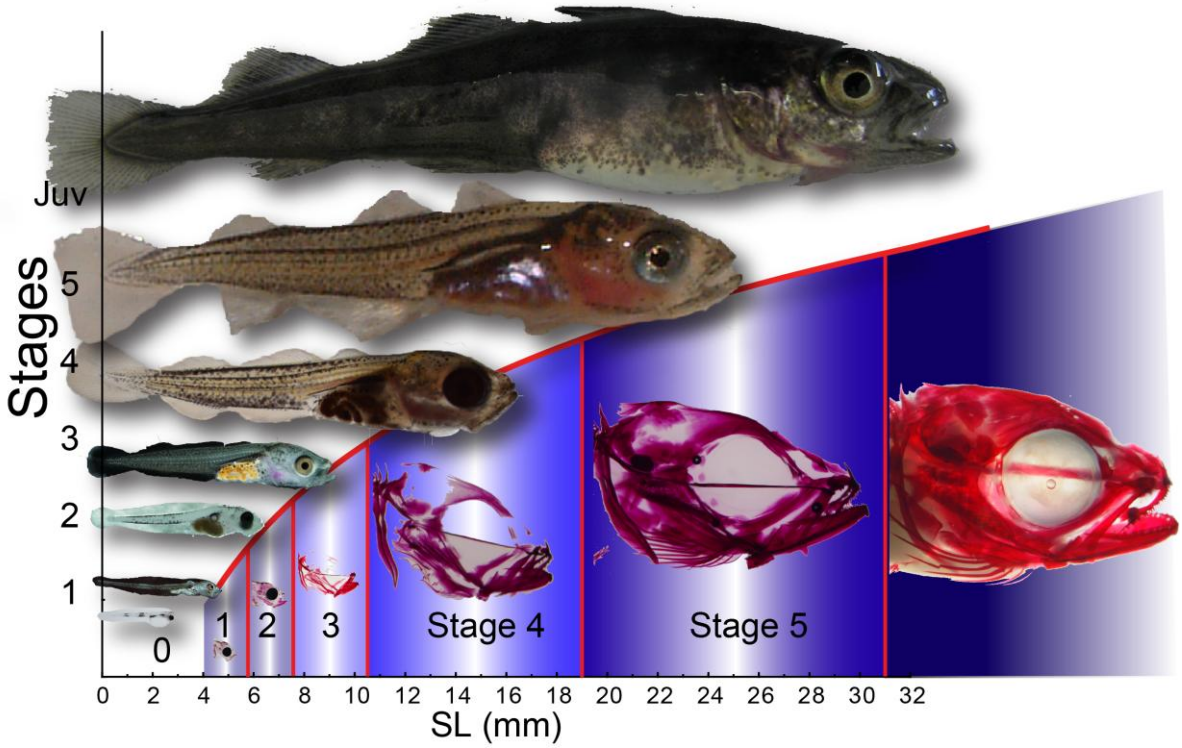

Figure 2

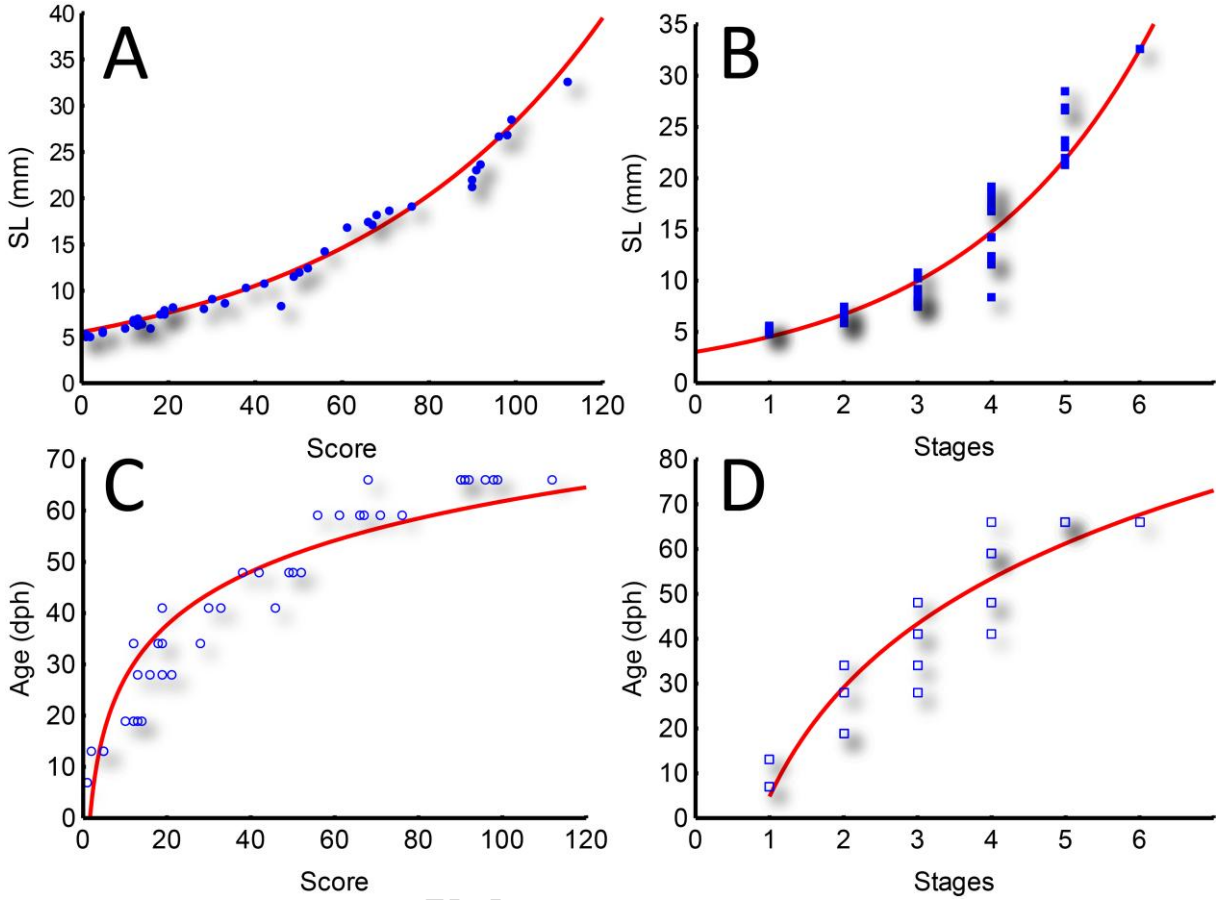

Figure 3

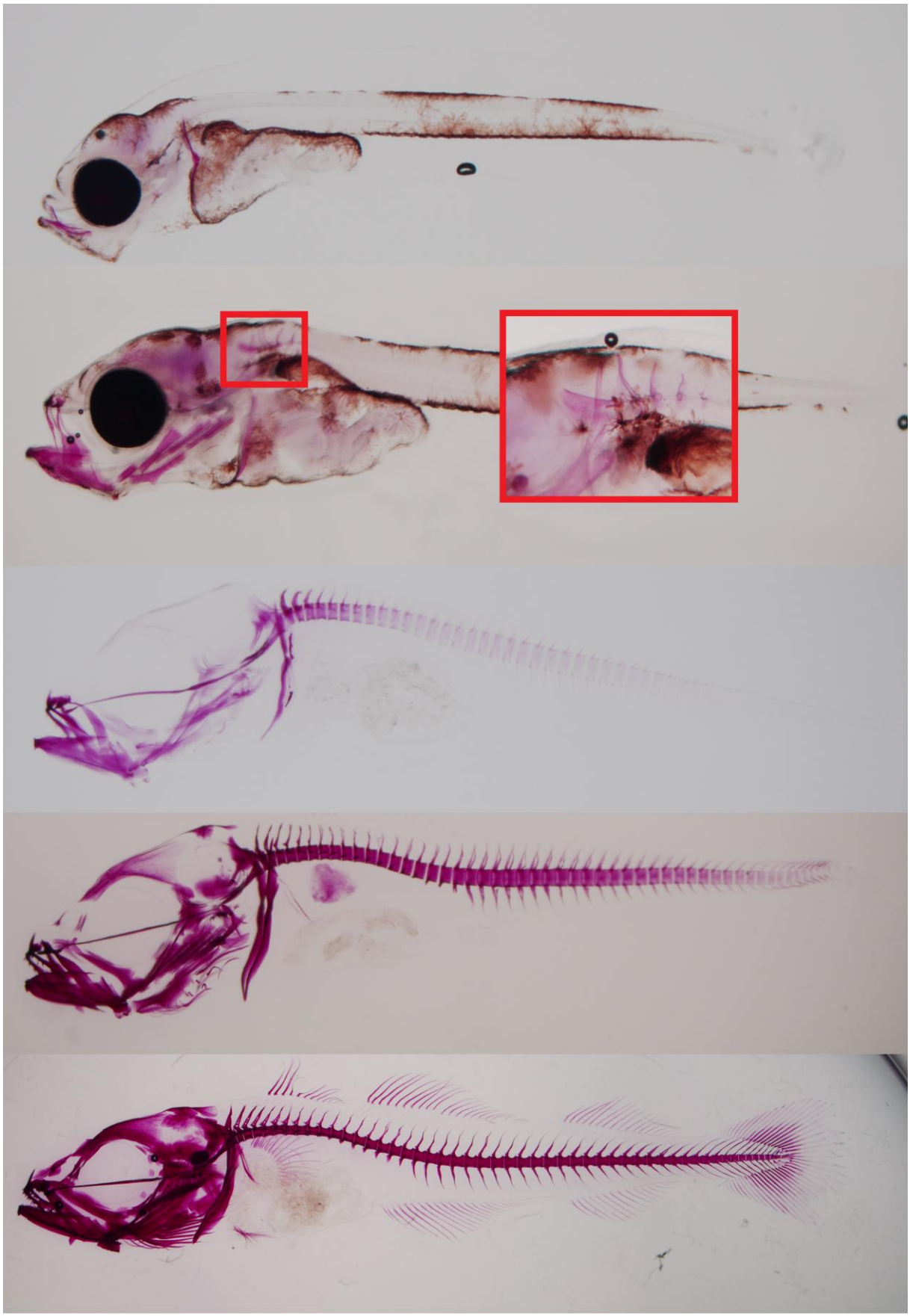

Figure 4

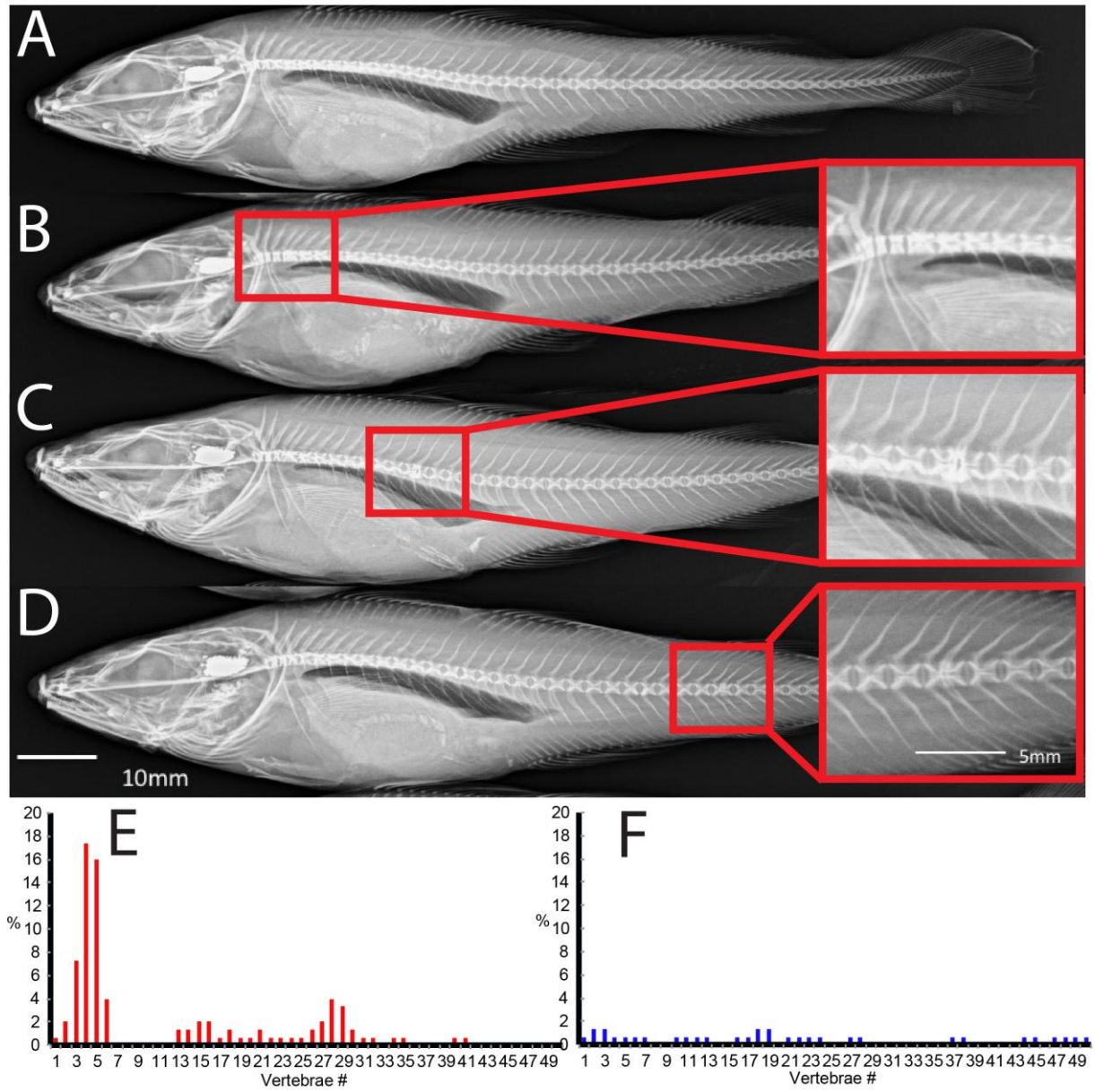

Figure 5

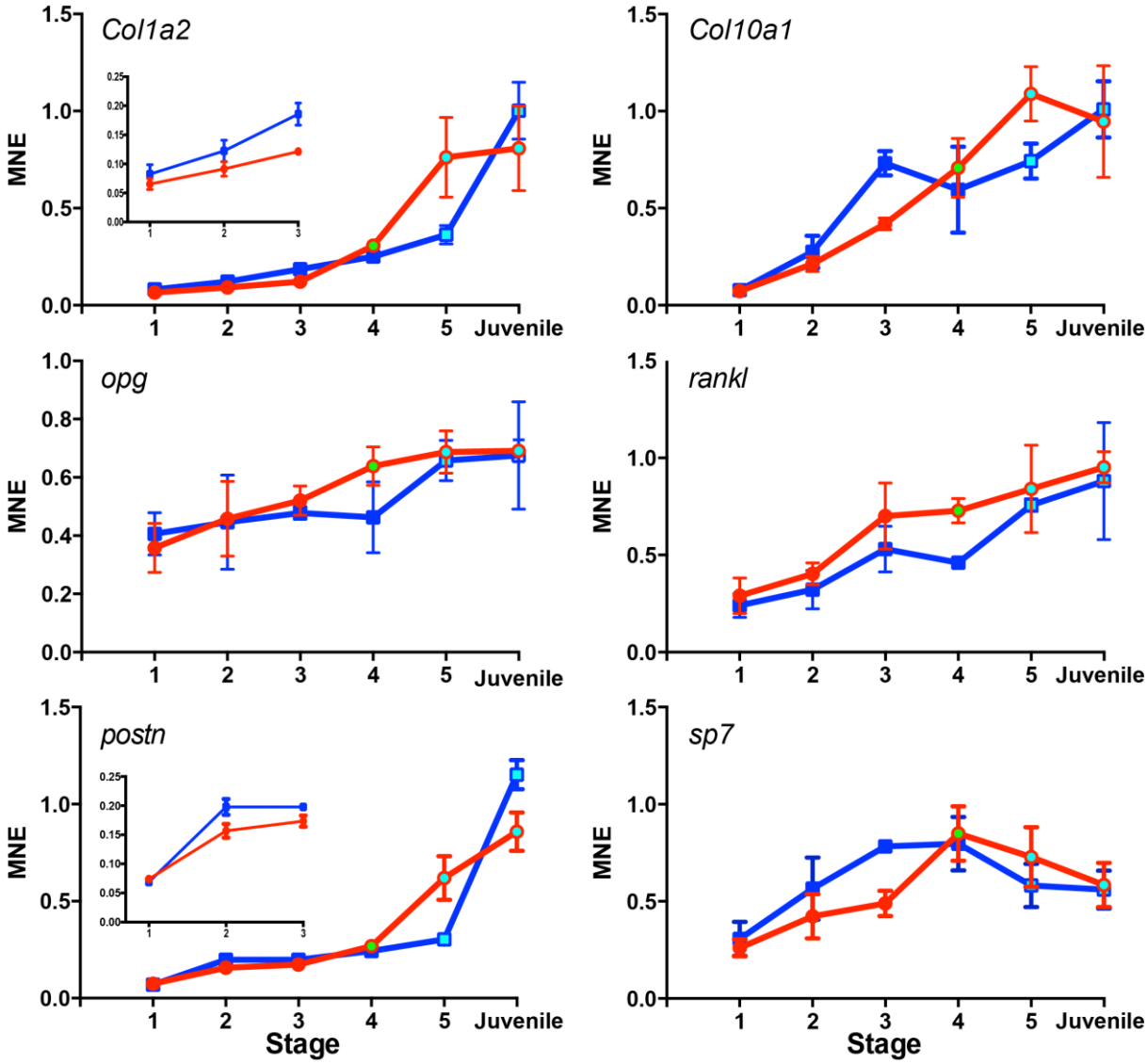

Figure 6

Table 1

	days post hatch		
Stage	Copepods	rotifer/Artemia	# of larvae
1	11	11	2000
2	22	22	840
3	29	31	160
4	37	54	31
5	53	71	20
6	74	85	20

Table 2

Gene symbol	Accession number	Forward primer	reverse primer	PCR efficiency	amplicon size
ubi	EX735613	GGCCGCAAAGATGCAGAT	CTGGGCTCGACCTCAAGAGT	1.9	69
bact	CO541508	CACAGCCGAGCGTGAGATT	ACGAGCTAGAAGCGGTTTGC	2.1	95
ctsk	HQ674762	GACATCAATCACGCCGTGTT	GCAGAGGTTGCCACGGTTAC	2.0	141
postn	HQ698962	GGTTACGACTGGGTCCCTTT	CAGGGTCATCCAAGGAGAGA	2.1	133
mgp	ENSGMOT00000011344*	CTTCTGACTGGGCTCCTGAC	TGTGGACCATCAAGTCTCCA	2.2	172
sp7	HQ674763	CCTCTGGCTATGCTAACCGC	AGGTCATCCCGTAGGCTTTCTT	2.2	121
bglap1	JQ861261	TTCTCCTCTTCCTCGCCTCA	TCTGTAGCAGCCCTCTTGGG	1.9	151
bglap2	JQ861262	TCTGCTGGACAACAGGAGTG	CGATGACGATGATGAGGATG	1.9	182
rankl	JK126260	GTCTACGCCAAGACCTGCAT	CGGACGTTCTCGTGGTAGAT	2.1	125
opg	JK126262	TGTGAAGCAGCACTGTACCC	CACCAGCTGCCTCTCCTTAC	2.0	133
col1a2	ENSGMOT00000014793*	CTACATGGACGCTGAGAGCG	CATTCGCCAGTGTGTGAAGTG	2.0	141
col10a1	ENSGMOT00000012206*	CTTCACCGTGTCTCTGGTCA	GGTCTCGGTGTCAAAGTGGT	2.0	100

Table 3

SL (mm)	5.7	5.7	5.7	5.13	5.13	6.13	6.19	7.19	6.19	6.19	6.28	7.34	7.28	7.34	8.34	8.41	7.28	8.28	8.34	9.41	9.41	8.41	10.48	11.48	12.48	12.48	12.48	14.59	17.59	17.59	18.66	17.59	19.59	19.59	21.66	22.66	23.66	24.66	27.66	27.66	29.66	33.66
Age (dpsf)																																										
Premaxillary							1	1	1	1	1	1	1	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	3	3	3	3	3	3	3	3
Maxillary		1	1	1	1	1	1	1	1	1	1	1	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	3	3	3	3	3	3	3	3
Palatine																												2	2	2	2	2	2	2	3	3	3	3	3	3	3	3
Mesopterygoid																							1	1	1	1	1	2	2	2	2	2	2	2	3	3	3	3	3	3	3	3
Ectopterygoid																			1	1	1	2	1	1	1	1	1	2	2	2	2	2	2	2	3	3	3	3	3	3	3	3
Quadrate															1	1	1	1	1	1	1	2	1	1	2	2	1	2	2	2	2	2	2	2	3	3	3	3	3	3	3	3
Articular							1	1	1	1	1	1	1	2	2	1	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	3	3	3	3	3	3	3	3
Angular										1	1	1	1				1		1	1	2	2	1	1	1	1	1	2	2	2	2	2	2	2	3	3	3	3	3	3	3	3
Dentary				1	1	1	1	1	1	1	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	3	3	3	3	3	3	3	3
Hyoid arch																																										
Hyomandibular												1	1						1		2	2	1	2	1	2	2	2	2	2	1	2	2	2	2	2	2	2	2	2	2	2
Symplectic							1	1	1	1	1	1	1	1	1	1	1	1	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2
Interhyal																																		1	2	2	2	2	2	2	2	2
Epihyal																									1	1	1	2	1		1	2	2	2	2	2	2	2	2	2	2	2	2
Ceratohyal							1	1	1	1	1	1	1	1	1	1	1	1	1	1	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2
Hypohyal											1	1	1	1	1	1		1	1	1	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2
Urohyal																				1			1	1	1	1	1	2	2	2	2	2		2	2	2	2	2	2	2	2	2
Operculum																																										
Opercular																				1		1	1	1	1	1	1	1	2	2	2	2	2	2	2	2	2	2	2	2	2	2
Praeopercular																				1		1	1	1	1	1	1	1	2	2	2	2	2	2	2	2	2	2	2	2	2	2
Subopercular																						1		1	1	1	1	1	2	2	2	2	2	2	2	2	2	2	2	2	2	2
Interopercular																						1	1	1	1	1	1	1	2	2	2	2	2	2	2	2	2	2	2	2	2	2
Neurocranium																																										
Nasal																								1	1	1	1	1	1			1	1	2	2	2	2	2	2	2	2	2	2
Ethmoid group																													1			1	1	1	1	1	1	2	2	2	2	2
Frontal																					1	1		1	1	1	1	1	1	2	2	2	2	2	2	2	2	2	2	2	2	2
Parteial																						1	1	1	1	1	1	1			1	1	1	1	1	1	1	1	2	2	2	2
Vomer																		1	1	1	1	1	1	1	2	2	2	1	2	2	2	2	2	2	2	2	2	2	2	2	2	3
Parasphenoid								1	1	1	1	1	1	1	1	1	1	1	2	2	2	2	1	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	3
Basisphenoid														1	1	1	1	1	2	2	2	2	2	1		1	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	3
Pterosphenoid																											2	1															
Pterotic																						1			1	1	1	1	1	1	1	1	1	1	2	2	2	2	2	2	2	2
Epiotic																																								1	1	1
Opistotic																																								1	1	1
Supraoccipital																													1	2	2	2	2	2	2	2	2	2	2	2	2	2
Exoccipital									1	1	1	1	1			1		1	1	1	1	1	2	2	2	2	2	1	1	1	1	1	2	2	2	2	2	2	2	2	2	2
Basioccipital								1	1	1	1	1	1	1	1	1	1	1	2	2	2	2	2	2	2	2	2	2	1	1	2	2	2	2	2	2	2	2	3	3	3	3
Orbital																																			(2)	(2)	(3)	(4)	(6)	(5)	(6)	(7)
Brancials																																										
Epibranchial																														1(4)	1(3)	1(4)	1(4)	1(4)	1(4)	1(4)	2(4)	2(4)	2(4)	2(4)	2(4)	2(4)
Ceratobranchial						1(3)	1(3)	1 (4)	1(4)	1 (4)	1 (4)	1 (4)	1 (4)	1 (4)	1 (4)	1 (4)	1(4)	1(4)	1 (4)	1 (4)	1 (4)	1 (4)	1 (4)	2(5)	2(5)	2(5)	2(5)	2(5)	2(5)	2(5)	2(5)	2(5)	2(5)	2(5)	2(5)	2(5)	2(5)	2(5)	2(5)	2(5)	2(5)	2(5)	3(5)
Hypobranchial																									1(1)	1(1)	1(1)	1(1)	1(1)	1(2)	1(2)	1(2)	1(3)	1(3)	1(3)	1(3)	1(3)	1(3)	1(3)	1(3)	1(3)	1(3)	2(3)
Basibranchial																																	1	1	1	1	1	1	1	1	1	1
Score	1	1	2	5	5	10	12	13	14	16	12	13	18	19	19	19	21	28	30	33	38	42	46	49	50	52	56	61	66	67	68	71	76	90	90	91	92	96	98	99	115

Statement of Relevance

This paper provides developmental stages that are vital for best practice protocols in aquaculture. By relating farming practices to developmental stages and not age, the right treatment ect may be provided.

This manuscript does also highlight the importance of nutrition during live feed stages on events that may occur late in the production cycle.

Highlights of the manuscript

We hereby present developmental stages for Atlantic cod

We also provide data on the best “easy to use proxy” to the stages, which is important for implementation in the industry

We demonstrate that nutrition during start feeding may impose weaknesses in skeletal elements that later in the juvenile/adult fish may result in deformations.