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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.

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.

1. 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 õembryo (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; Sæle et al., 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

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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).

2. Materials and methods

2.1. Sampling and analyses

Experiments and sampling were carried out at the Institute 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).

2.2. 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 >80%. 16,250 larvae were then transferred to the first feeding tanks (400 l, 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.

2.3. 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. Fig. 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).

3. 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 (C14H7O7SNa) (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

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

	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