Aquaculture International
https://doi.org/10.1007/s10499-018-0314-7

Effects of different incubation and start-feeding temperature regimes on growth, survival, and histomorphology of cod larvae

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Received: 28 May 2018 / Accepted: 17 October 2018/Published online: 25 October 2018
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Abstract

Mean lengths of newly hatched cod larvae from egg incubation at 4 °C (Low-L) and 9.5 °C (High-H) were similar, and only minor differences were observed in larval histomorphology. However, growth performance of larvae reared at 4 °C (T1-LL) and 9.5 °C (T2-LH) from the 4 °C egg incubation and 4 °C (T3-HL) and 9.5 °C (T4-HH) from the 9.5 °C incubation group were different during start-feeding and metamorphosis. Incubation and larval rearing temperature affected developmental rate and survival. T1-LL and T2-LH larvae were larger than T4-HH larvae at sampling stage 4 (early larvae), but the differences disappeared thereafter. Larvae from T3-HL did not survive beyond stage 8 (late larvae/start metamorphosis), and survival of T1-LL larvae at the end of the experiment was very low. Larvae from T2-LH were significantly larger than larvae from T4-HH at stage 12 (end metamorphosis). Comparative studies of the histomorphology of vital organs did not reveal temperature-related differences between sampled larvae/early juveniles. Characteristic traits in the histomorphology of cod larvae at the selected developmental stages are presented. Our results show that egg incubation and subsequent larval rearing temperature affected the growth performance and survival but did not have any significant effects on the organ development and histomorphology.

Keywords Atlantic cod larvae · Temperature · Development · Histomorphology · Survival

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Introduction

Fish egg and larval developmental rates, hatching success, growth, and mortality are highly influenced by temperature (Blaxter 1988, 1992; Johnston 1993; Folkvord 2005; Geffen et al. 2006). Although the basic developmental mechanisms of teleosts are similar, there are species-specific differences with respect to temperature tolerance and the timing of developmental events. Atlantic cod, *Gadus morhua* L., spawn and develop at relatively low temperatures, which vary geographically as well as seasonally during both egg and larval periods. Hatching success at temperatures from 2 to 12 °C has been found to decrease at the higher end of the scale (Laurence and Rogers 1976; Iversen and Danielsen 1984; Nissling 2004; Geffen et al. 2006; Jordaan et al. 2006). According to Kuftina and Novikov (1987), incubating cod eggs in the temperature interval 0–6 °C, the duration to hatching was nearly linear and the duration of developmental steps decreased with increasing temperature, while the relative duration of each step was changed. Many researchers have reported longer larvae at hatch when incubating the eggs at low temperatures (Pryor and Brown 1998; Peterson et al. 2004; Puvanendran et al. 2015). Experimental studies have also reported hatching of shorter larvae from very low egg incubation temperatures (Galloway et al. 1998). Fitzsimmons and Perutz (2006) found no differences in survival at hatch and higher prevalence of malformations in cod larvae from eggs incubated at temperatures in the range of 6–11 °C. Finally, Puvanendran et al. (2015) reported that a short step-wise increase in incubation temperature (from 4.5 to 9.5 °C) from the zygote stage influenced hatching rate as well as the percentage of deformed cod larvae.

The differentiation of cells into tissues, organs, and organ systems of fish proceeds continuously from the early embryonic phase throughout metamorphosis (Blaxter 1988). Ontogenetic organ system development during embryonic and larval stages of Atlantic cod has been studied and illustrated by, e.g., Kjørsvik et al. (1991), Pedersen and Falk-Petersen (1992), Morrison (1993), Hunt von Herbing et al. (1996a), Hunt von Herbing et al. (1996b), Galloway et al. (1998), Hall et al. (2004), Falk-Petersen (2005), and Puvanendran et al. (2015). However, most of these studies cover only the differentiation of selected organs or tissues during specific phases of the early life history. A comparative histological developmental status in relation to temperature increase throughout cod larval development is lacking.

The present work was included in the national cod-developmental project CODE (Research Council of Norway-199482) which was established to explain the mechanisms behind early life history success or failure of Atlantic cod. The investigation aimed at identifying survival, growth, and possible organ histological differences between larvae originating from eggs that were incubated at two different temperatures and raised under different temperature regimes.

Materials and methods

Cod broodstock and spawning

The broodstock that were used in this study were transferred to sea cages in Tromsø as 10-month-old second-generation juveniles and were kept in the sea for 2 years. One month prior to spawning, they were transferred to the Center for Marine Aquaculture (CMA) in Tromsø, North Norway (69°40′0″ N) and kept in 25-m^3 tanks receiving ambient temperature water. Temperature during this period ranged from 2 to 8 °C (whole year cycle) and was 4 °C during

spawning. Sperm from eight males and eggs from 18 females were hand-stripped. Gametes were pooled and fertilization was carried out according to Hansen and Puvanendran (2010).

Experimental setup

Fertilized eggs were transferred to sixteen 25-L upwelling incubators (375 ml eggs in each) and eight of the incubators were kept at 4 °C (± 0.2 °C; ambient temperature), while temperature was gradually increased to 9.5 °C (± 0.2 °C) in 32 h in the other eight incubators (Puvanendran et al. 2013). At 100% hatch, larvae were transferred to 190-L circular fiber glass tanks at a density of 136 larvae L^{-1}. Larvae from eggs incubated at 4 °C were transferred to eight tanks at 4 °C, while those from 9.5 °C were transferred to eight tanks at 9.5 °C. Of the former, temperature in four tanks was kept at 4 °C (T1-LL), while the temperature in the other four tanks was gradually increased to 9.5 °C from 5 days post-hatch (dph) to 10 dph (T2-LH). Of the latter, temperature in four tanks was gradually decreased to 4 °C from 5 to 10 dph (T3-HL), while the other four were kept at 9.5 °C (T4-HH). Thus, four different experimental groups were established to study temperature effects on development, survival, and growth during the larval and early juvenile stages (for more details, see Bizuayehu et al. 2015; Chauton et al. 2015). One of the replicate tanks from T4 had unexpected high mortality on day 4 of the experiment, while the other three tanks had low mortality. Thus, only three tanks were used for sampling and growth measurement in T4.

Larval rearing

Water flow was maintained at 1.1 L min^{-1} at 2 dph and gradually increased to 3.3 L min^{-1} at 56 dph. Microalgae (*Nannochloropsis* sp., Reed Mariculture, USA) were added to the tank twice a day during the first 10 days. Larvae were start-fed with nutrient-enriched rotifers 10 times a day (PhosphoNorse® and MicroNorse®, Trofi AS, Tromsø) from 2 to 29 dph at a density of 4000 rotifers L^{-1}. Rotifer and *Artemia* suspension in water at different temperatures were not monitored; however, the constant aeration and water flow would have aided to keep the live organisms in the water column. Rotifer feeding was gradually replaced by enriched *Artemia* (enriched with Multigain®, MicroNorse®, and PhosphoNorse®) from 25 to 29 dph. Larvae were fed six times a day with enriched *Artemia* from 30 to 37 dph, and a co-feeding strategy was implemented from 38 to 44 dph where dry feed was added to the tanks 1 h before *Artemia* feeding. Larvae were weaned onto dry feed (200–300 and 300–500 μm, AlgoNorse Coldwater®, Trofi AS, Tromsø) from 45 to 56 dph by withdrawing the *Artemia* in five steps. The above feeding regime changes were delayed by 2–3 weeks for the T1 group because of slower development and thus smaller size at these ages.

Larval sampling

Due to expected differential growth at different temperature regimes, sampling of larvae was standardized using developmental stages (modified from von Herbing et al. 1996a) rather than age. Larval samplings were conducted at five predetermined stages; stage 1/2 (newly hatched), stage 4 (early larval stage—sub-ventral mouth, 10–25% yolk left, rounded non-functional swim bladder, maximally elevated supra-cephalic sinus), stage 8 (late larvae/start metamorphosis—pronounced upper jaw, one small loop between mid-gut and intestine, enlarged functional swim bladder, collapsing supra-cephalic sinus), stage 11 (mid-metamorphosis—

stomach formed, more coiled intestine, start of vertical swim bladder elongation, sinus completely collapsed, well-formed caudal fin rays, pre-curser to dorsal fins), and stage 12 (end metamorphosis—fully elongated silvery swim bladder, distinct three dorsal fins, mini adult form). All replicates representing the same temperature regime were sampled simultaneously. Ten larvae per tank (40 per treatment) were sampled for growth measurements at each sampling point. Further, ten more larvae were sampled randomly from each replicate, anesthetized with MS-222, transferred to 4% phosphate-buffered formaldehyde, and embedded in Technovit and/or Paraplast for comparative histological studies of organ and tissue development. Longitudinal sections of five larvae from each sample were cut on a rotation microtome, were stained with toluidine blue (Technovit-embedded larvae) or hematoxylin/eosin (paraplast-embedded larvae/juveniles), and were studied and photographed in a Leitz Aristoplan microscope with a Leica DFC295 camera.

Surviving juveniles from each treatment (T1-LL, T2-LH, and T4-HH) were counted at 90 dph; T3 (HL) did not survive until this point.

Growth data was analyzed using a two-way ANOVA and a Bonferroni-corrected post hoc test employed when a significant difference was detected. Residuals were examined for the assumption of independence, homogeneity, and normality (Sokal and Rohlf 1995). The significance level was set at 0.05.

Results

No significant differences between mean lengths and weight at 100% hatch (stage 1/2) were found between the larvae hatched from eggs incubated at 4 and 9.5 °C ($F = 0.666$, $p < 0.46$; and $F = 0.5$, $p < 0.519$, respectively). Temperature had, however, a significant effect on the growth of cod larvae after the onset of exogenous feeding ($p < 0.001$). Larvae from T1-LL ($p < 0.042$) and T2-LH ($p < 0.004$) were significantly larger than larvae from T4-HH at stage 4, but the differences disappeared thereafter (Fig. 1). At stage 12, larvae from T2-LH were significantly larger than larvae from T4-HH ($p < 0.001$). Time to reach any specific developmental stage was clearly affected by temperature at incubation and larval rearing. Larvae from T1-LL ($p < 0.0001$) were the slowest to reach stages 8 and 11 compared to larvae from T2-LH and T4-HH (T1–29 and 60 dph; T2–27 and 43 dph; T4–17 and 42 dph, respectively). Larvae from T3-HL reached stage 8 at 23 days and did not survive beyond this point. Larvae from T4-HH reached stage 12 significantly earlier ($p < 0.0001$) than T2-LH (57 and 66 dph, respectively). Survival of larvae from T1-LL was significantly lower compared to T2 ($p < 0.025$) and T4 ($p < 0.043$) after 90 days (0.7, 11.4, and 9.7%, which corresponds to 175, 2850, and 2425 juveniles, respectively). Larvae from these experiments were planned to be carried through juveniles and to adults, and due to low survival in the T1 tanks, sampling was stopped at stage 11.

Histological sections of hatched larvae from the high and low egg incubation temperature regimes did not reveal pronounced differences with regard to organ or tissue histomorphology (Fig. 2a, b). At this stage (100% hatch), the larvae had a relatively large yolk sac, cartilaginous jaw apparatus elements, and pigmented eyes, a compact swimbladder without lumen, a premature heart, and no circulating blood cells, four primitive cartilaginous epithelia-covered gill arches, and no gill lamellae (Fig. 2a, b). Larvae from the high incubation temperature had a more developed branchial artery lumen in the gill arch epithelium layer (Fig. 2b, G) compared to those incubated at the low temperature. Numerous chloride cells lined the gill slit epithelium and pharynx and appeared in the subpharyngeal region too, above the heart. The digestive tract was slightly differentiated; the foregut

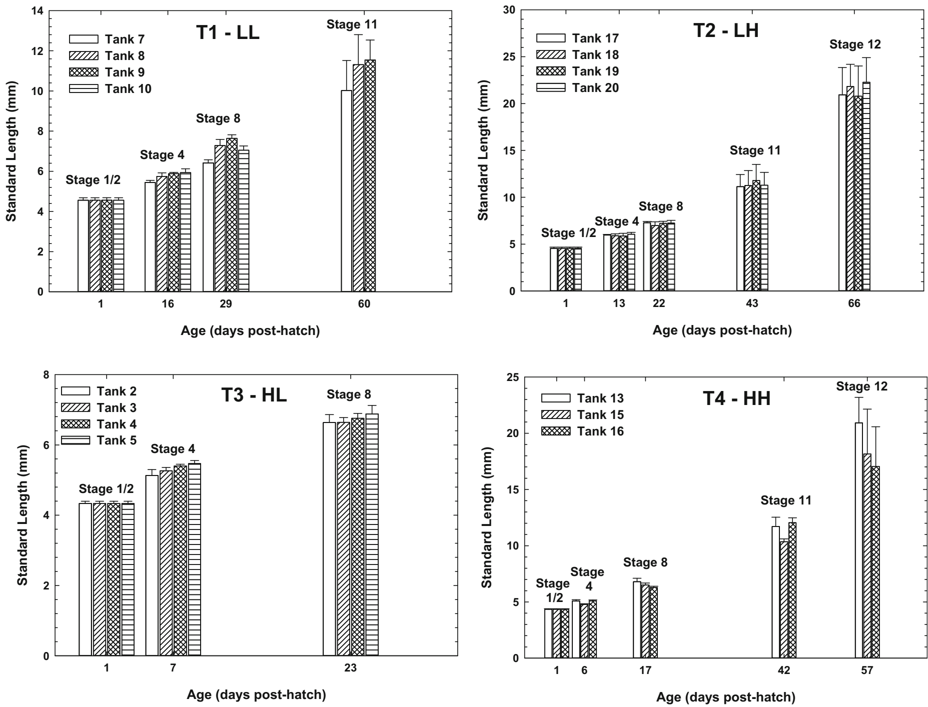

Fig. 1 Lengths and age of larvae at hatch, stage 4 (early larvae), stage 8 (late larvae/start metamorphosis), stage 11 (mid-metamorphosis), and stage 12 (end metamorphosis) from the various incubation and feeding temperature regimes (T1 = LL, T2 = LH, T3 = HL, and T4 = HH)

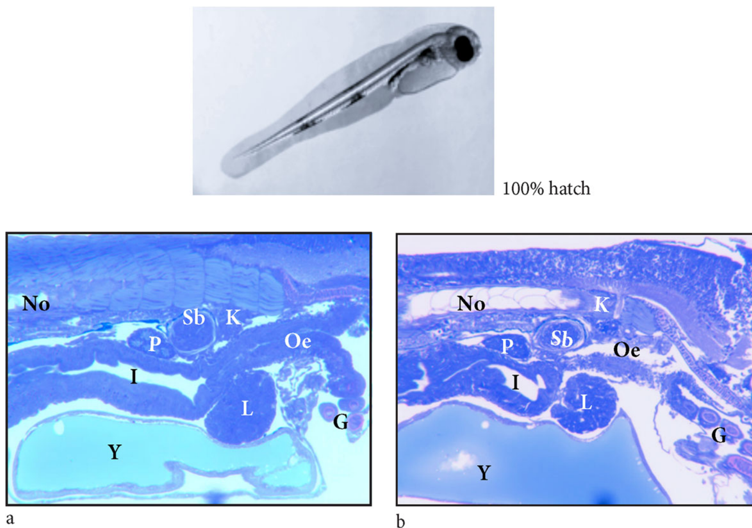

Fig. 2 **a**, **b** Longitudinal sections of hatched cod larvae from stage 1 (hatched) (× 80). (Common legends to Figs. 2, 3, 4, 5, and 6: A = atrium, B = bulbus arteriosus, E = eye, G = gill arch/gills, I = intestine, K = kidney, L = liver, No = notochord, Oe = esophagus, Ol = olfactory organ, Ot = otolith, P = pancreas, Pc = pyloric caecae, Sb = swim bladder, St = stomach, Tf = thyroid follicles, V = ventricle)

(esophagus) with thick mucosa and narrow lumen and at this point no visible mucous cells and a slightly folded midgut with larger lumen, clearly separated from the hindgut (Fig. 2a, b). The liver was globular with a compact hepatocyte structure, a thick-walled gallbladder was present, and pancreatic tissue was prominent (Fig. 2a, b). A urinary bladder was also established and so was the pronephros with tubuli, a glomerulus and hematopoietic/lymphomyeloid tissue.

Organs and tissues of larvae from the four temperature regimes sectioned at stage 4 (first-feeding initiated) also appeared similar (Fig. 3a–e). The gut was full of feed in most larvae. Thyroid-follicles were detected in all samples, located in the subpharyngeal region, lining the ventral aorta (Fig. 3e). Intestinal differentiation and folding had increased, and a few mucous cells were now lining the esophagus (foregut) mucosa. A lumen was established in the

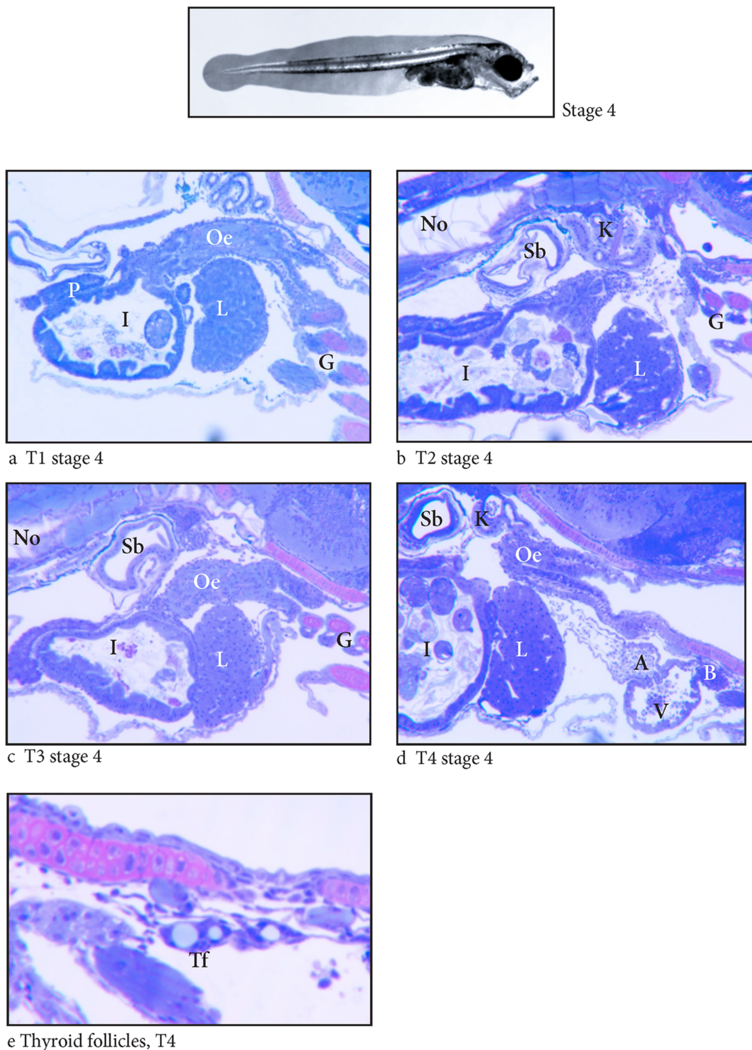

Fig. 3 **a**–**e** (**a**–**d** × 80, **e** × 200). Longitudinal sections of cod larvae from stage 4 (early larvae) from T1, T2, T3, and T4

swimbladder (Fig. 3b–d), and blood cells were present in the circulatory system (Fig. 3d). Elongation of epithelial tissue surrounding the gill arches indicated initial formation of gill primary filaments and blood cells were noted in the branchial arteries of larvae from all groups.

Increased intestinal convolution and number of mucous cells in the esophagus characterized stage 8 larvae (late larvae/start metamorphosis) (Fig. 4b–f), and the liver had increased in size but less in structure (no vacuolization of the hepatocytes) (Fig. 4a–f), while pancreatic tissue had expanded. Short gill filaments were established (Fig. 4d) and the slightly oblong swimbladder had a large lumen (Fig. 4b, e). The T3 group suffered high mortality after stage 8 as mentioned before, but the larvae sectioned until then had apparently normal histomorphology (Fig. 4e, g). Thyroid follicles were prominent (Fig. 4c–h).

An early stomach was apparent in stage 11 larvae (mid-metamorphosis) and the hepatocytes had become vacuolized (Fig. 5a–d). Intestinal loops and gut area had expanded dramatically. Gill filaments were longer (Fig. 5a, d), the heart appeared well-developed (Fig. 5a), and the swimbladder was generally elongated, although some variations were noted within groups with regard to actual length.

The full expansion of the swimbladder was similar in T2 and T4 larvae at stage 12 (late metamorphosis) (Fig. 6a). The gill filaments (both primary and secondary) were long, and the heart appeared fully differentiated (Fig. 6d, e). At stage 12, the digestive tract histology of the T2 and T4-groups revealed a large stomach with digestive glands and numerous pyloric caeca (Fig. 6a–c). Teeth buds and taste cells were noted in the pharynx. A characteristic vacuolized liver histomorphology was present from stage 11, but even more pronounced in all stage 12 larvae (Fig. 6a–c). At this stage, the olfactory organ was enclosed in the nares region (Fig. 6f) and the axial skeleton differentiation/mineralization had started.

Discussion

Larvae originating from eggs incubated at low and high temperature had rather similar growth and developmental rates when reared at higher temperature (T2-LH and T4-HH), whereas larvae reared at low temperature from both groups (T1-LL and T3-HL) performed poorly. The reduction of larval rearing temperature in T3-HL after hatching became lethal after stage 8. Larval survival of T1-LL was poor, and later, juveniles from this group showed increased and severe skeletal deformities (data not shown).

Larval lengths were not significantly different between hatchlings from eggs incubated at 4 and 9.5 °C, in contrast to findings from an earlier rearing experiment (Puvanendran et al. 2015) when cold water larvae were found to be longer. Literature reported that effects of incubation temperature on larval size of Atlantic cod are not consistent, however. Pryor and Brown (1998) found that total length at hatch and at EYS (end-of-yolk-sac-stage) of cod was highest at low incubation temperatures (0–1 and 3–4 °C compared to 7–8 °C), while larval dry weights were not significantly different. According to Galloway et al. (1998), cod larvae hatching from eggs incubated at 1 °C were shorter than those from 5 and 8 °C, while survival was similar in the three groups. Mean lengths of newly hatched cod larvae at incubation temperatures from 4 to 10 °C were greater at 4 °C according to Peterson et al. (2004), while Jordaan et al. (2006) reported that length at hatch was influenced by incubation temperature (2–12 °C) in only two of three cod egg batches. The latter result could explain the variations reported in the literature and the apparent larval length similarity in our experiment which was carried out with a mixture of eggs from several females. Experiments with other cold-water fish species having larger and more yolky eggs have reported

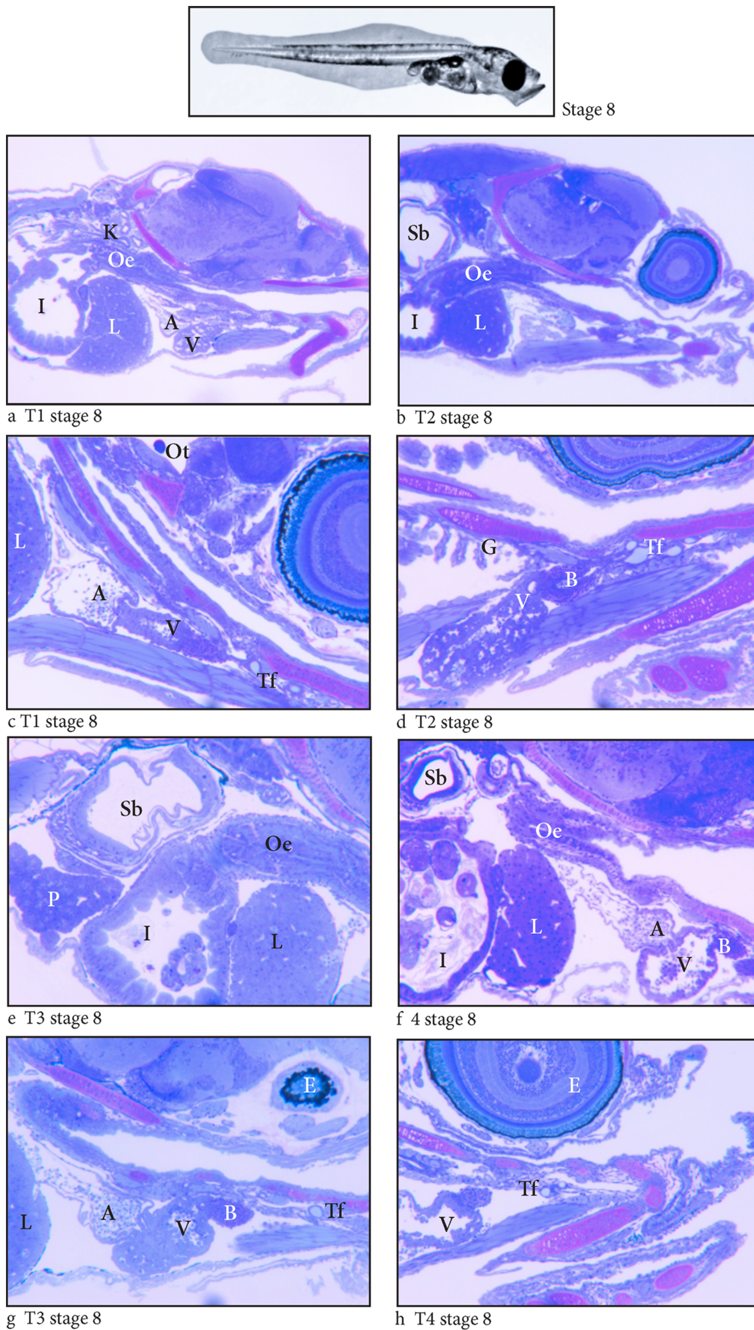

Fig. 4 **a**–**h** (**a**, **b**, **e**, **f** × 80, **c**, **d**, **g**, **h** × 200). Longitudinal sections of cod larvae from stage 8 (late larvae/start metamorphosis) from T1, T2, T3, and T4

larger larvae with less yolk reserves at hatch at lower egg incubation temperatures (Falk-Petersen 2005; Sund and Falk-Petersen 2005; Danielsen 2016).

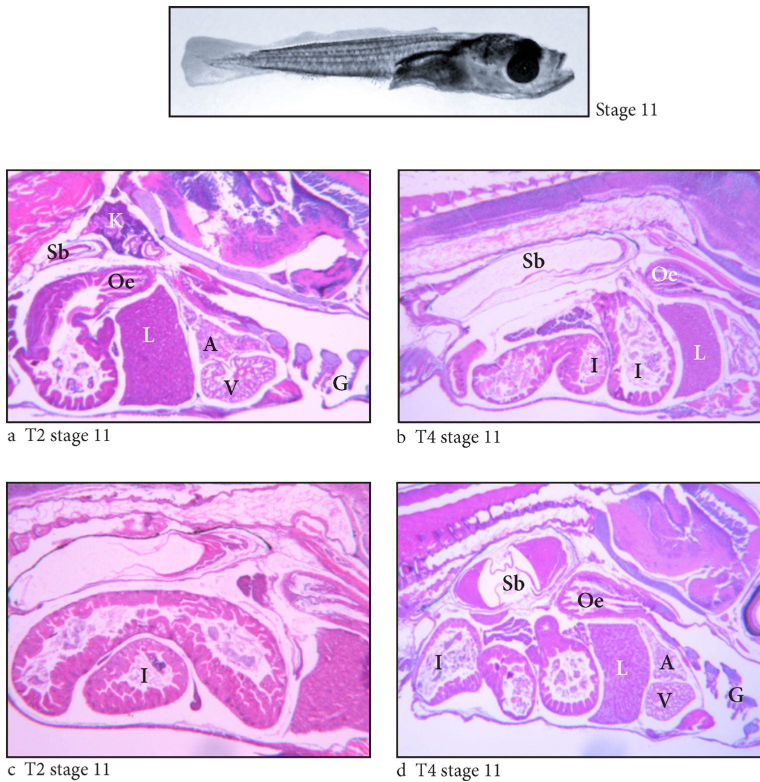

Fig. 5 **a**–**d** (× 80). Longitudinal sections of cod larvae from stage 11 (mid-metamorphosis) from T2 and T4

Ontogeny of organ development and its associated physiological functions are species-specific and directly affected by several abiotic and biotic factors, such as temperature and prey availability during early life stages. Early life history environment may influence the performance of fish at later life stages too (Jonsson and Jonsson 2014); the mechanisms behind these effects are not clear though. Detailed comparative studies of organ and tissue histomorphology might reveal functional differences or abnormalities among larval groups from different temperature regimes. However, in our experiment, larvae from all temperature regimes generally showed similar overall histomorphology at the selected sampling stages. A minor difference was noted between hatched larvae from eggs incubated at 4 and 9.5 °C: the latter showing slightly more developed branchial artery differentiation. This could be the result of the higher temperature speeding up developmental processes or that temperature affects the development of various organs in different ways (Hunt von Herbing et al. 1996a). Using samples originating from the same large study (CODE) as ours, Bizuayeshu et al. (2015) showed that temperature increase during embryonic and/or early larval stages can alter the microRNA (miRNA) profile and suggested that this alteration could have short- and long-term effects on the Atlantic cod. Another study from CODE using similar material (Chauton et al. 2015) reported that temperature changes during early stages of cod development influenced the larvae at the metabolomic level. Although changes were detected at the molecular and metabolomic levels and on growth

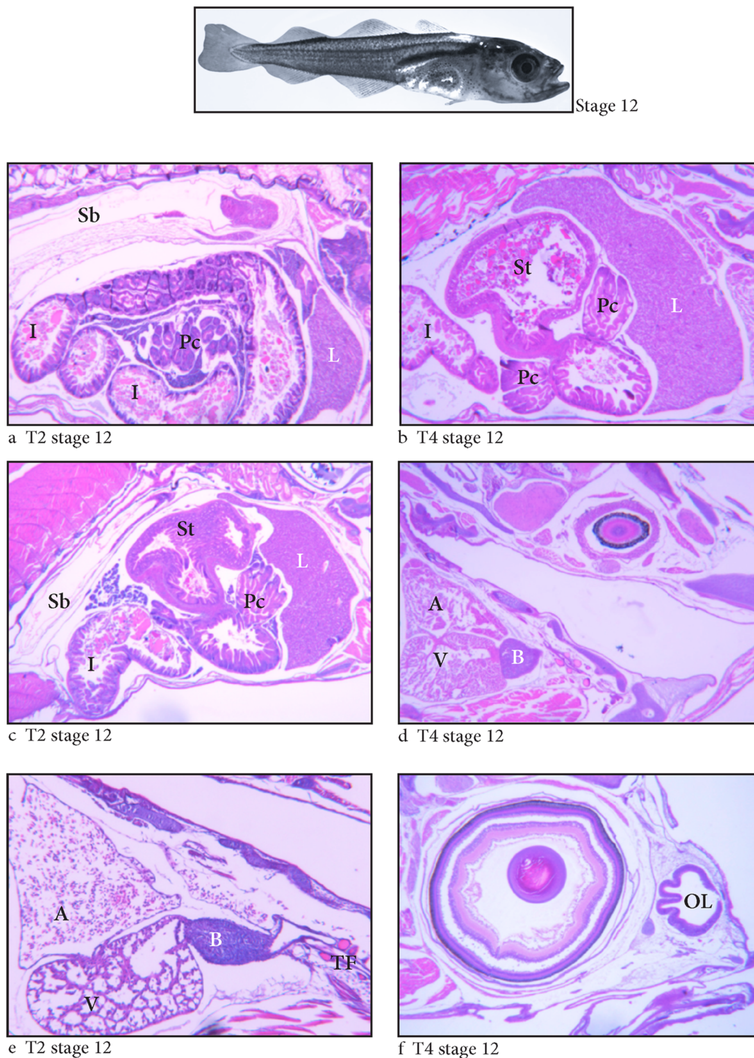

Fig. 6 **a**–**f** (**a**–**c** × 80, **e**, **f** × 200). Longitudinal sections of cod juveniles from stage 12 (end metamorphosis) from T2 and T4

and survival among different temperature regimes, no apparent changes were observed at tissue or organ level during larval development and metamorphosis. At this point in time, it is not possible to explain this disparity among different biological levels. Even if the T3-HL regime was detrimental to the larvae and mortality surprisingly high (99.3% mortality from start of the experiment to 90 days post hatch) in the T1-LL regime, no obvious organ or tissue abnormalities were detected in the sections compared to the T2 and T4 groups. At stage 12 (end metamorphosis), the sampled juveniles had a mature organ histomorphology, even if parts of the digestive channel (e.g., the pyloric caeca) had not yet reached the adult relative proportions (Pedersen and Falk-Petersen 1992).

Conclusion

Temperature manipulations during embryonic and larval stages of Atlantic cod did not elicit noticeable histological changes in any organs. This contrasts with the reported changes in mRNA (genomic level) and metabolomics (physiological level) and performance data (growth and survival) from the same experiment. There is an apparent lack of connection between different biological mechanism levels in relation to the thermal experience of embryos and early larvae of Atlantic cod.

Acknowledgments We would like to thank the staff at the Center for Marine Aquaculture Research in Tromsø for support during the experiment and Lara Agulló Núñez and Fride Tonning at the histolab at the Institute of Arctic and Marine Biology, University of Tromsø, for sectioning and staining of numerous larvae. This investigation was financially supported by the Research Council of Norway (CODE, RCN-project 199482).

Compliance with ethical standards

Conflict of interest The authors declare that they have no conflict of interest.

Ethical approval All applicable international, national, and/or institutional guidelines for the care and use of animals were followed by the authors.

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