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Hormonal control of tryptic enzyme activity in Atlantic cod larvae (*Gadus morhua*): involvement of cholecystokinin during ontogeny and diurnal rhythm

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Abstract

The ontogenetic development of the gut hormone cholecystokinin (CCK) and the key proteolytic enzyme trypsin was described in Atlantic cod larvae (*Gadus morhua*) from first-feeding until 38 days post first-feeding (dpff). CCK is known to play a major role in the endocrine control of digestive processes in mammals and adult fish, but its regulatory role in the larval stages of marine fish is largely unknown. Only small amounts of CCK were found in the body (excluding head) in cod larvae at first-feeding, but CCK levels increased exponentially with development, suggesting a more pronounced role of CCK during ontogeny. Tryptic enzyme activity increased slightly until a standard length of ca. 8 mm (approx. 33 days dpff) with a significant increase in larvae larger than 8 mm standard length, indicating limited digestive capacity in the early stages. To entangle the short-term feedback mechanism between CCK and tryptic enzyme activity, we conducted a 12 hour feeding experiment at 21 dpff. Cod larvae receiving only algae revealed a noticeable response in tryptic enzyme activity within two hours in the morning, whereas larvae fed algae and rotifers at the same time showed a slightly delayed response up to four hours. Tryptic enzyme activity remained low in the group receiving only algae as well as the two fed groups in the afternoon. No reaction in tryptic enzyme activity was observed in larvae that received a second meal of rotifers in the afternoon, indicating limited regulatory and digestive capacity to handle several meals in a short period. CCK levels remained relatively constant throughout the day but increased in the afternoon in all three groups when tryptic enzyme activity was low, suggesting that a negative feedback mechanism between CCK and tryptic enzyme activity is present in larval cod at least from 21 dpff.

Key words: Atlantic cod larvae, trypsin, CCK, digestion, ontogeny, endocrine control

1. Introduction

The unstable and unpredictive production of juveniles of many marine fish species for aquaculture still prevents commercialization of many candidate species. One reason is the lack of knowledge of the function and efficiency regarding digestive physiology in the early stages that hampers formulation of proper feeds and feeding regimes. There is a good understanding of how the tissues and organs of the digestive system develop during larval fish ontogeny. Accordingly, the developmental gene expression and secretion patterns of the digestive enzymes have been well described (reviewed by Lazo et al., 2011). However, research on the endocrine control of digestive functions in fish larvae is still in its infancy (Webb and Rønnestad, 2011) and also lags behind that of adult fish (e.g. Murashita et al., 2008). Consequently, models derived from mammals and adult fish in most cases serve as starting points to discover similar mechanisms in developing fish larvae.

Until altricial fish larvae acquire an adult-like mode of digestion, characterized by a fully functional stomach including gastric glands and acidic digestion, they rely mainly on serine proteases with trypsin-like enzymes as the most significant proteolytic enzymes in the early larval stages. These alkaline proteases are synthesized in the pancreas and secreted into the gut, following the ingestion of feed. Among these enzymes, trypsin is considered to be a key enzyme in the digestive process (Zambonino Infante and Cahu, 2001). Trypsin is secreted as its inactive precursor trypsinogen from the acinar cells of the pancreas into the gut lumen and either auto-activated or activated by the enzyme enteropeptidase. In marine fish larvae, the amount of tryptic enzyme activity in the gut has been demonstrated to be a function of feed ingestion, gut filling and the composition of nutrients (Rønnestad and Morais, 2007; Ueberschär, 1995).

The gastrointestinal hormone cholecystokinin (CCK) is known to play a key role in contraction of the gallbladder, peristalsis in the intestine, delay of gastric emptying and pancreatic enzyme secretion in mammals (Silver and Morley, 1991) and adult fish (Einarsson et al., 1997). In addition, it acts as a satiation signal in the fish brain (Volkoff et al., 2005). In mammals, CCK is considered one of the most important stimulators of pancreatic enzyme secretion (Liddle, 2006) and is therefore an obvious candidate to

study the same functions in larval fish. Upon the presence of nutrients in the gut, CCK is released from enteroendocrine cells in the gut epithelium into the body fluids and acts on target cells in the pancreas to release secretions into the gut lumen. The stimulation in the gut might be of mechanical and/or biochemical nature. High tryptic enzyme activity in the gut acts as a negative feedback control for the release of CCK in humans, suggesting a regulatory loop between these two factors (Liddle, 2006) and the same mechanism has been described in adult fish (Murashita et al., 2008). The spatial distribution of the CCK-producing cells in the larval gut seems to vary between fish species (e.g. Kamisaka et al., 2005; Webb et al., 2010), and knowledge of the regulatory mechanism between CCK and trypsin still remains limited in developing fish larvae. Moreover, the differences in the spatial and temporal appearance of these cells indicate species-specific differences in controlling digestive processes (Rojas-García et al., 2011). Additionally, it has been shown in mammals that certain food components and digestive end products, like intact protein or certain amino acids, stimulate CCK and consequently pancreatic enzyme secretion, more than other nutrients (Liddle, 1995). Results of controlled tube-feeding studies (Koven et al., 2002), as well as standard feeding trials (Cahu et al., 2004; Naz and Türkmen, 2009), suggest that similar mechanisms are present in early larval stages of fish. Low amounts of these stimulatory components in commercial microdiets may contribute to the inability of most marine fish larvae to utilize these diets efficiently from first-feeding (Yúfera et al., 2000).

Although functional studies on daily rhythms in marine fish larvae exist (e.g. feed uptake; Kotani and Fushimi, 2011; Pedro Cañavate et al., 2006), physiological studies on digestive processes are not widespread in the literature and focused mostly on the response of proteolytic enzyme activity in relation to feeding schedules (Applebaum and Holt, 2003; MacKenzie et al., 1999; Ueberschär, 1995). Nevertheless, knowledge of diurnal cycles of physiological aspects, including digestive processes, has gained some attention in recent studies (Fujii et al., 2007; Harboe et al., 2009; Rojas-García et al., 2011; Yúfera, 2011). They may provide insight, for instance, on feeding times, feeding amounts and number of meals in larviculture practices in relation to digestive capacities and endogenic rhythms of the larvae.

Aquaculture of Atlantic cod (*Gadus morhua*) is a relatively young industry with many challenges, including larviculture. Moreover, as wild cod stocks are being highly exploited in the North Atlantic and there are many unknown factors involved in the recruitment and mortality of year classes, this species serves as a model organism to tackle basic questions in larval digestive physiology. Here, we describe the ontogenetic development of CCK and tryptic enzyme activity in Atlantic cod larvae to provide insights into the capacity to regulate digestive processes in early cod. We conducted a short-term feeding experiment to evaluate the interaction between CCK and tryptic enzyme activity over 12 hours following different numbers of meals. Previous studies have shown that there is a relatively large amount of CCK found in the head part (central nervous system mainly) which may mask changes of CCK in the gastrointestinal tract (Rojas-García et al., 2011). Therefore, all analyses in the present study were done on dissected larvae excluding the head.

Apart from disclosing the postulated mechanisms of CCK and tryptic enzyme activity, this provides valuable information on the diurnal digestive capacity in early cod related to practical feeding conditions.

2. Materials and Methods

2.1 Larval rearing

Fertilized cod eggs were incubated in a 75 L hatching incubator for 17 days with full-strength seawater at 5.5 - 5.9°C. Gentle bubbling from the bottom kept the eggs in suspension and the water was exchanged at 4 L min^{-1} to maintain optimal water quality. Newly hatched cod larvae (3 days post-hatch) were counted using density estimates of three tube samples and 50000 larvae were transferred to a first-feeding tank (450 L) to establish a density of around 110 larvae L^{-1}. The black feeding tank was equipped with a two-directional water inlet immediately below the water surface at 50% of the tank radius and was aerated with fine bubbling using an aeration ring in the middle bottom of the tank. Water flow was gradually increased from 0.6 L min^{-1} on day 1 of the experiment (1 day post first-feeding, dpff) to 3.0 L min^{-1} at the end of the experimental period (38 dpff). Oxygen remained between 93 - 99% saturation throughout the experiment. Water temperature was gradually increased from 6°C to 11°C. Light was provided 24 hours a day applying indirect illumination of the rearing room and a weak light bulb (100 lux) above the tank. Dead larvae and debris were removed daily by siphoning the tank bottom and by using an automatic and rotating cleaner arm later in the experiment. A surface skimmer was installed to keep the water surface clean.

Microalgae *Nannochloropsis* sp. paste (Nanno 3600, Reed Mariculture, USA) was pre-mixed with seawater and added daily in the morning (10 ml, 1 - 16 dpff; 15 ml, 17 - 38 dpff). Enriched rotifers (*Brachionus plicatilis*; LARVIVA Multigain, BioMar, Denmark) were administered twice a day in the morning (10:00) and afternoon (15:00) with increasing rotifer densities in the tank throughout the experiment (5 rotifers ml^{-1} to 30 rotifers ml^{-1}). In addition, algae paste and rotifers were provided continuously after the second feeding using a separate storage tank and a peristaltic pump. Enriched *Artemia* instar II *nauplii* (EG Artemia, INVE, Belgium) were co-fed with rotifers at densities of 1 ml^{-1} from 32 dpff until the end of the experiment. The larval rearing followed the best larviculture practices at the Austevoll Research Station.

10 - 15 larvae were sampled randomly each sampling day prior to feeding in the morning using a pipette with a large opening. The larvae were transferred with minimum

of seawater into an Eppendorf vial and then immediately frozen on dry ice and subsequently stored at -80°C until analysis.

2.2 Short-term diurnal rhythm experiment

A 12 hour experiment was conducted in seven, green 40 L tanks on 21 dpff. Each tank was equipped with a water inlet right below the water surface and was aerated the same way as the main black tank described above. Water flow was set to 0.3 L min^{-1} and water parameters (Temperature, O_2%) were not different from the main black tank. One day in advance, 150 larvae were transferred to each tank after the second meal in the afternoon for acclimatization.

On the day of the experiment, 1.25 ml algae paste was added to each tank at 9:30 and 15:15, respectively. Three tanks were fed enriched rotifers once at 10:30 ("one meal") at a density of 22 rotifers ml^{-1} and another three tanks were fed twice at 10:30 and 15:30 ("two meals") at densities of 22 rotifers ml^{-1} and 11 rotifers ml^{-1}, respectively. One tank was only given algae paste (considered as the "control" group). 5 - 10 larvae were sampled hourly as quickly as possible from each tank between 8:00 - 20:00 as described above and stored at -80°C until analysis.

2.3 Sample preparation

Individual samples were analyzed for CCK and tryptic enzyme activity according to Rojas-García et al. (2001) and Ueberschär (1993). Described briefly, frozen samples were allowed to thaw on ice, rinsed with distilled water and the standard length (mm, tip of upper jaw to end of notochord) was measured on an ice-cold petridish under a microscope. Gut fullness was evaluated, using a simple gut fullness index after Rojas-García *et al.* (2011): 0% (empty), <25%, 25 - 50%, 50 - 75%, 75 - 100% (full). The head was dissected from each larva and excluded from the analyses. Each larva was then transferred to an individual Eppendorf vial and homogenized in 50 µl ice-cold distilled water using a motorized pestle. For extraction of CCK, 750 µl Methanol were added, the sample was vortex-mixed thoroughly and incubated on ice for 30 min. After centrifugation (15 min., 1700 g, 4°C) each sample was split in two by transferring the

supernatant to a new Eppendorf vial. Both, the remaining pellet (methanol-insoluble fish precipitate) and the supernatant (CCK methanol extract) were evaporated to dryness using a vacuum desiccator attached to a water-jet pump and stored at -20°C until analysis.

2.4 Analysis of CCK and tryptic enzyme activity

The individual CCK extracts were assayed by a competitive radioimmunoassay (RIA) using CCK-RIA kits (RB302, Euro-Diagnostika, Sweden) according to the supplier's instructions and Rojas-García et al. (2001). CCK levels were interpolated from a standard curve (0.78 - 25 pmol CCK L^{-1}) and concentrations are expressed as fmol $larva^{-1}$. Recovery of known amounts of CCK added to samples throughout the extraction procedure was 71%.

Tryptic enzyme activity in individual pellets was measured using a highly specific fluorescence substrate (Nα-benzoyl-L-arginine-methyl-coumarinyl-7-amide-HCl) according to Ueberschär (1993). Values for tryptic enzyme activity are expressed as hydrolysed fluorescence products MCA (methyl-coumarinyl-7-amide, nmol MCA min^{-1} $larva^{-1}$). The coefficient of variation between triplicate measurements of samples was 1.6% (n = 4 samples).

2.5 Statistical analysis

CCK concentrations and tryptic enzyme activity levels during ontogenetic development were averaged for length classes in steps of 0.5 mm (4.0 – 4.5, 4.5 – 5.0 etc.). Data of the diurnal rhythm experiment were tested for normality and homogeneity of variance using the Shapiro-Wilks-test and Levene's test, respectively. Significant differences in standard length, gut fullness, CCK concentration and tryptic enzyme activity were analyzed using a nested One-way ANOVA with measurements of individuals in each tank nested in treatment groups for each sampling point. Upon significance, differences between groups were assessed with Student-Newman-Keul's test. Significant differences between sampling points within each fed group were analyzed using a nested One-way ANOVA followed by a post hoc Duncan's multiple-comparison test.

Values of gut fullness were transformed using the formula *gut fullness'=arcsin√gut fullness*. Statistics were performed with SPSS 19.0 for Windows and the level of significance was set to $p < 0.05$. Data are presented as mean ± SD.

3. Results

3.1 Ontogeny

The mean standard length of cod larvae was 4.59 ± 0.24 mm on 1 dpff and increased to 8.14 ± 0.41 mm at the end of the experimental period (38 dpff, Fig. 1). Growth was gradual during the first 3 weeks but tended to stagnate between 23 and 30 dpff.

CCK was present in the dissected body at the earliest developmental stage and increased exponentially with increasing larval standard length (Fig. 2). Tryptic enzyme activity increased slightly until the larvae reached a standard length of approx. 8 mm with a sharp increase afterwards and ranged between 0.04 and 54.35 hydrolysed MCA, nmol min^{-1} with increasing variability over time among individuals of comparable size (Fig. 3).

3.2 Short-term diurnal rhythm experiment

The observations of the gut fullness over the 12 hour feeding period are given in Fig. 4 for all three groups. Ingestion of rotifers is indicated by an immediate increase in gut fullness both in the “one meal” and “two meals” group as short as 30 min. after feeding in the morning. Mean levels of gut fullness remained relatively stable over the whole day in both fed groups with the values in the “two meals” group being slightly higher compared to the “one meal” group. Gut fullness in the “control” group refers to aggregation of algae trapped in the hindgut in some of the individuals and was generally lower compared to larvae of the two fed groups with significant differences (except 12:00 and 17:00) compared to the two fed groups after feeding in the morning. Highly significant differences in gut fullness between samplings points within groups were found before (10:00) and immediately after feeding (11:00) in the one-meal group ($F_{12,26}$ = 5.187; $p < 0.0001$) as well as in the two-meals group ($F_{12,26}$ = 18.914; $p < 0.0001$). The standard length was 7.09 ± 0.46 mm, 7.34 ± 0.57 mm and 7.32 ± 0.52 mm for the “control”, “one meal” and “two meals” group, respectively. The larvae were not significantly different in standard length between all three groups at any sampling point (data not shown).

CCK levels increased between 9:00 and 14:00 in all groups with fluctuating levels afterwards until the end of the sampling period (20:00). The highest levels were recorded one hour earlier at 19:00 with 2.69 ± 1.52 fmol larva^{-1}, 1.89 ± 0.16 fmol larva^{-1} and 1.94 ± 0.22 fmol larva^{-1} in the “control”, “one meal” and “two meals” group, respectively (Fig. 5). This time point represents 8.5 hours after the addition of rotifers in the “one meal” group and 3.5 hours after the second addition in the “two meal” group. The lowest levels were recorded one hour later at the end of the sampling period at 20:00 with 0.70 ± 0.33 fmol larva^{-1}, 0.75 ± 0.15 fmol larva^{-1} and 0.58 ± 0.27 fmol larva^{-1} in the “control”, “one meal” and “two meals” group, respectively. CCK levels were not significantly different between groups at all sampling points.

Tryptic activity in the “control” group increased gradually with a marked drop at 11:00 which was 1.5 hours after the addition of algae (1.89 ± 1.41 hydrolysed MCA nmol min^{-1} larva^{-1}, Fig. 5), reaching a peak at 12:00 (7.51 ± 3.70 nmol min^{-1} larva^{-1}) and decreased successively between 12:00 and 19:00, irrespective of the second addition of algae at 15:15. The development of tryptic activity showed almost similar patterns in the “one meal” and the “two meals” groups. After an initial increase in the morning, tryptic enzyme activity dropped at 11:00 in both groups, 30 min. after the addition of rotifers (3.55 ± 0.56 nmol min^{-1} larva^{-1} for “one meal” and 4.07 ± 1.64 nmol min^{-1} larva^{-1} for “two meal” larvae). 4.5 hours after the administration of rotifers tryptic activity increased to reach a peak at 15:00 (7.51 ± 1.76 nmol min^{-1} larva^{-1} for “one meal” and 7.15 ± 2.73 nmol min^{-1} larva^{-1} for “two meal” larvae). Tryptic enzyme activity leveled off afterwards in both fed groups reaching lowest levels at 20:00 9.5 hours after the addition of rotifers (2.10 ± 0.81 nmol min^{-1} larva^{-1}) in the “one meal” group and 19:00 3.5 hours after the second addition of rotifers (2.60 ± 1.24 nmol min^{-1} larva^{-1}) in the “two meal” group (Fig. 5). No significant differences in tryptic enzyme activity were found between all groups at any sampling point.

4. Discussion

4.1 Ontogeny

We conducted the present study to examine the ontogenetic development of CCK and tryptic enzyme activity in cod larvae between 1 - 38 dpff. An additional aim was to examine the dynamic dependency of both factors based on a 12 hour monitoring of CCK and tryptic enzyme activity related to a different number of meals.

Growth expressed as standard length showed a gradual increase in the experimental period and was comparable to growth reported in recent literature on cod larvae (Busch et al., 2011; Meyer et al., 2012; Penglase et al., 2010). A noticeable growth depression was evident between 23 - 30 dpff. In this period, a disease referred to as the “Distended Gut Syndrome” appeared, which symptoms have been described in several marine fish larvae, including cod, and causes cumulative mortality due to loss of appetite (Kamisaka et al., 2010). Around 20 - 30% of the larvae sampled in this period revealed signs of DGS and this might have been the reason for reduced growth. These larvae had generally lower gut fullness and tryptic enzyme activity values (data not shown). All larvae with signs of DGS were excluded from further statistical analysis.

As mentioned previously, it has been shown that CCK is quantitatively dominant in the brain of larval fish, e.g. as described for herring (*Clupea harengus*)(Rojas-García et al., 2011), halibut (*Hippoglossus hippoglossus*)(Rojas-García and Rønnestad, 2002) and sea bass (*Dicentrarchus labrax*)(Tillner et al., unpubl. data). Besides its regulatory role of the digestive processes, CCK also acts as a satiation signal in the brain of humans (Smith, 2009) and adult fish (Volkoff et al., 2005). However, the latter role has not been experimentally explored in larval fish. In the present study, our aim was to investigate the role of CCK in relation to the regulatory functionality on tryptic enzyme activity in the gut. Consequently, the head of all cod larvae was separated before the analyses in order to exclude neural sources of CCK. However, it must be pointed out, that no differentiation between synthesized CCK in cells in the gut epithelium and released CCK into the body fluids was made in the present study. CCK was found in larval cod immediately after hatching, although these concentrations were close to the detection limit of the RIA, proposing a limited regulatory function of CCK at this developmental

stage. The following exponential increase of CCK in larval cod over standard length suggests an increasing importance of CCK in the digestive system which is supported by data that demonstrated an increasing number of CCK-producing cells during ontogeny using immunohistochemical staining from 6 dpff onwards (Hartviksen et al., 2009). These cells were mainly found in the anterior midgut where contact with stimulatory nutrients most likely permits a regulation of gallbladder and pancreas secretions but also in the hindgut later in development where the physiological role is less clear. A pronounced individual variability in the number of CCK-producing cells within comparable developmental stages was revealed, although the authors pointed out that different postprandial states or the low sensitivity of the staining method might have contributed to this variability (Hartviksen et al., 2009). Nevertheless, individual differences in CCK-producing cells might explain the variability of CCK levels in the present study. In general, data on the ontogenetic development of CCK content in fish larvae are rather scarce and most studies focused on the description of CCK-producing cells (Hartviksen et al., 2009; Kamisaka et al., 2001; Kamisaka et al., 2005; Kamisaka et al., 2003; Micale et al., 2010; Webb et al., 2010), gene expression of CCK (Kortner et al., 2011a; Kortner et al., 2011b) or both (Kurokawa et al., 2000; Kurokawa et al., 2004). Kortner et al. (2011b) revealed a moderate but consistent decrease in CCK expression in whole larval cod between 3 - 60 days post hatch. Given the assumption that CCK is mainly expressed in the brain, the authors contribute the decreased expression to a decreasing proportion of brain tissue compared to the whole body. This statement is supported by a study from Cahu et al. (2004) on larval sea bass in which a clear allometric relationship between dry weight specific CCK content and dry weight was evident. In contrast, a study on larval sea bream (*Sparus aurata*), using the same analytical method for CCK as in the present study, revealed almost no increase in whole body CCK until 40 days post-hatch in pooled samples (Naz and Türkmen, 2009). To reveal the ontogenetic development of CCK in more detail, Rojas-García and Rønnestad (2002) described the compartmental distribution of CCK in first feeding halibut larvae (*Hippoglossus hippoglossus*) between 7 - 26 dpff, where the proportion of CCK in the gut compared to whole body CCK increased from 2 - 62%. In contrast, a

different distribution of CCK was found in larval herring (*Clupea harengus*) where whole body CCK increased 15-fold until 40 days post-hatch, but CCK in the gut remained constant at relatively low levels (Rojas-García et al., 2011). This difference between halibut and herring could be contributed to morphological and consequently physiological differences of the digestive tract, with herring having a straight and halibut having a rotated gut (Rojas-García et al., 2011). However, it remains to be proven, if these low amounts are able to elicit a regulatory response in first-feeding fish larvae (Rojas-García and Rønnestad, 2002) which also counts for the CCK concentrations found in the present study in larval cod.

With regard to tryptic enzyme activity, only a slight increase could be observed in our study until the larvae reached a standard length of 8 mm (approx. 33 dpff), which is the same pattern found for trypsin by Kortner et al. (2011b). On the transcriptional level, an increased mRNA expression for trypsin until 17 days post-hatch was observed by these authors, followed by a continuous decline thereafter. This discrepancy between gene expression and enzyme activity could be contributed to a post-transcriptional hormonal control or to a developing acidic digestion due to a developing stomach towards metamorphosis (Kortner et al., 2011b). In the earlier case, CCK levels between 0.5 - 1.0 fmol larva^{-1} found for larval cod of comparable age (to 17 dph in Kortner et al., 2011b) in the present study might be the threshold for the regulatory role of CCK in larval cod. In general, the developmental pattern of different digestive enzymes has been described in several species, including California halibut (*Paralichthys californicus*)(Alvarez-González et al., 2005), sea bass (Cahu and Zambonino Infante, 1994), cobia (*Rachycentron canadum*)(Faulk et al., 2007), red drum (*Sciaenops ocellatus*)(Lazo et al., 2000a) and Atlantic cod (Wold et al., 2007) and has been found to be species-specific. It has been proposed, that digestive enzyme activities in early stages of marine fish larvae are under transcriptional control, but can be triggered by the nutritional composition of the diet (Zambonino Infante and Cahu, 2001). In our study, a decrease in tryptic enzyme activity is obvious between 7.5 - 8.0 mm standard length (approx. 25 - 35 dpff) and might indicate only limited digestive capacity during this developmental period in larval cod. This intermediate decrease has been observed in several species

and has been proposed as ontogenetic deficiencies in digestive capacity and the inability to digest food properly at certain developmental stages (Ueberschär, 1995). This assumption is strengthened by the fact, that trypsin is by far the most important enzyme for protein digestion in the larval stages of altricial species (Ueberschär, 1995). For example, a decrease in digestive capacity has been observed in larval herring two weeks after first-feeding with copepods, independent of food density (Pedersen et al., 1990). Moreover, certain diets might be inadequate to provoke a digestive response via the CCK-trypsin axis, as the decrease in tryptic enzyme activity in our study coincides with the transitional feeding period from rotifers to *Artemia*. Species-specific differences and a more rapid development in digestive capacity may, at least in parts, contribute to the success in larviculture of different species (Ueberschär, 1995). Furthermore, individual variability in tryptic enzyme activity at comparable developmental stages of one species may divide individual larvae into “winners” and “losers” and might explain individual fates in survival and growth.

4.2 Short-term diurnal rhythm experiment

In order to evaluate the suggested feedback mechanism between CCK and tryptic enzyme activity in dependence on a different number of meals, we conducted a short-term experiment at 21 dpff. The immediate increase in gut fullness in the “control” group after the addition of algae in the morning suggests an active or passive uptake of algal cells which are trapped in the hindgut (Kjørsvik et al., 1991). Addition of algae (“green water”) is routinely used in larviculture and is believed to aid in water quality, to enhance contrast for capturing prey (Conceição et al., 2010; Rocha et al., 2008; van der Meeren et al., 2007) and to reduce stress. Ingested algae were, however, not considered to represent a full meal in terms of energy and nutrients supplied. In the morning, live rotifers were immediately ingested by the larvae, indicated by increased mean gut fullness 30 min. after the addition in the two fed groups. The degree of gut fullness in those two groups was more or less constant over the sampling period and was only slightly higher in the “two meal” group at almost all sampling points. However, there was no clear effect of a second feeding in the afternoon, indicated by the gut fullness of the

"two meal" group. Although the rotifer density in the tanks was not evaluated, a complete water exchange every 2 - 3 hours and the consequent removal of rotifers indicates a gut retention time of ingested prey up to several hours in cod larvae at this age. CCK increased slightly in all groups until 14:00 without clear differences in all groups. A marked increase in all groups was revealed at 19:00 reaching highest levels in all groups followed by a drop one hour later reaching lowest levels in all groups which points to a delayed response to a nutrient stimulus of newly ingested algae or algae and rotifers. The only study on diurnal rhythm of CCK in fish larvae was a recent work on Atlantic herring done by Rojas-García et al. (2011). In their investigations, no clear response of CCK in the body (excluding head) was observed after feeding over three days, although CCK levels were higher in fed larvae compared to starved larvae. An increase in the carcass/gut ratio of CCK 30 min. after feeding can be contributed to a release and re-synthesis of CCK (Rojas-García et al., 2011). Similarly, an increase in the carcass/gut ratio of CCK was also found in tube-fed halibut larvae four hours after the administration of protein (albumin) as a nutrient stimulus (Rojas-García and Rønnestad, 2002). This underlying effect might be masked in the present study, since whole-body homogenates, excluding heads, were analysed.

Tryptic enzyme activity started from relatively high levels in the morning prior to feeding times in all three groups, although almost no larvae had food remainings in their gut at this time. This can be considered as the pre-feeding basal level of regularly fed larvae. Similarly, larvae of herring and Japanese eel (*Anguilla japonica*) have been shown to retain trypsin in their intestine up to several hours after a meal to be available for newly arriving prey (Pedersen and Andersen, 1992; Pedersen et al., 2003). On the other hand, it has been shown that adult sea bream are able to synchronize their behavior and increase enzyme secretion to a fixed feeding time to prepare for a forthcoming meal (Montoya et al., 2010). In addition, a diurnal rhythm in feed ingestion and tryptic enzyme activity over three days was observed by Fujii et al. (2007) for malabar grouper larvae (*Epinephelus malabaricus*, 3 - 5 days post-hatch) despite continuous light and stable prey densities in the rearing tanks. Similar reasons might be responsible for the relatively high tryptic enzyme activity in the morning in larval cod. Interestingly, a

marked decrease in tryptic enzyme activity was evident at 11:00 in all groups. Since trypsin is retained in the gut and not reabsorbed for up to several hours after a meal (Pedersen and Andersen, 1992), this marked decrease in tryptic enzyme activity might be the consequence of immediate binding of trypsin to algal cells and rotifers acting as a substrate. There was no real starving group represented in the present study, and the increase in tryptic enzyme activity at 12:00 in the “control” group is most likely the consequence of ingested algae that trigger the digestion process, as proposed by other authors (Cahu et al., 1998; Reitan et al., 1997). An additional group without algae and rotifers could have shed light on the role of algae in triggering digestive processes and also on graded responses in the digestive system to the size and type of ingested particles/prey (Hjelmeland et al., 1988). On the long term, the addition of algae might facilitate the maturation of the digestive system by triggering digestive processes and might contribute to the overall higher success of larviculture in “green-water” described in the literature (e.g. Cahu et al., 1998; Faulk and Holt, 2005; Lazo et al., 2000b). Consequently, rearing marine fish larvae in “green-water” might be an essential part of best larviculture practices. The peak in the “control” group was followed by a gradual decrease towards the end of the day, indicating an immediate release of trypsinogen from the pancreas after nutritional stimulation followed by empty stores afterwards and basal tryptic enzyme activity levels in the gut. The increase in tryptic enzyme activity was delayed up to three hours in the two fed groups, reaching high levels at 15:00, respectively. This might be due to slow and gradual release of stimulatory components from the ingested rotifers although based on visual inspection of cod in the present study, the rotifers were rapidly degraded. The fact that maximum tryptic enzyme activity levels were not different between the two fed groups and the group receiving only algae (control) may imply a dominant role of a chemical stimulus over a mechanical stimulus of ingested prey. Alternatively, only slight mechanical stimulation by the algae might be sufficient to elicit a digestive response. In contrast, the content of trypsin in the gut is found to be a function of ingested prey items in larval herring (Pedersen et al., 1987). Similarly to the “control” group, tryptic enzyme activity tended to drop in the afternoon in both fed groups with no apparent effect of a second meal in the “2 meals” group. This

observation strengthens the indication, that pancreatic resources for trypsinogen in larval cod are exhausted after an initial stimulation with no response after subsequent ingestion of algae or algae and rotifers. This also emphasizes the importance of retaining and re-using trypsin in the intestinal lumen.

In contrast, tryptic enzyme activity was persistently high in larval herring after two consecutive sequences of feeding and digestion, pointing to the ability of larval herring to digest several meals within a short period after an initial release of enzymes (Pedersen and Hjelmeland, 1988). Similar results were shown for larvae of African catfish (*Clarias gariepinus*) seven days post first-feeding, where, given several meals a day, tryptic enzyme activity fluctuated around a stable level over 24 hours, indicating a higher capacity to handle several meals a day in this species (García-Ortega et al., 2000). Similar to the present study, a decrease in tryptic enzyme activity 1 - 2 hours after food ingestion was found in the catfish, indicating an immediate utilization of trypsin in the gut for protein hydrolysis. Therefore, a response in tryptic enzyme activity to a food stimulus after ingestion might be reflected thereafter (García-Ortega et al., 2000). A delayed postprandial response in tryptic enzyme activity has also been shown for larval turbot (*Scophthalmus maximus*), with a persistent postprandial increase in older larvae, indicating an increasing digestive capacity with age in these species (Ueberschär, 1995).

Taken together, CCK and tryptic enzyme activity revealed a reverse diurnal trend in all groups in the present study, with a marked increase in CCK when tryptic enzyme activity levels were low. Given the high and stable gut filling over the entire sampling period in all groups which most likely results in a continuous and stable nutrient stimulus in the gut, the data suggest that CCK is synthesized and released as a stimulatory response when tryptic enzyme activity in the gut is low and vice versa. When this regulatory loop becomes functional still remains to be established. Such negative feedback control has been observed in humans (Liddle, 2000) and has been proposed to be also effective in fish larvae (Cahu et al., 2004; Rønnestad et al., 2007). In detail, the presently available data suggest that in the presence of trypsin a CCK-releasing peptide in the gut is degraded, whereas ingested protein competes as a substrate for

trypsin and the intact CCK-releasing peptide stimulates the release of CCK (Liddle, 1995; Miyasaka et al., 1989). In a controlled tube-feeding study on first-feeding herring larvae, Koven et al. (2002) found an immediate increase in whole-body CCK after administration of solutions containing protein (albumin), protein plus free amino acid and free amino acid only (in descending order of CCK response). This was followed by an immediate increase in tryptic enzyme activity in all groups (in the same order of magnitude). Given the failed postprandial response in body-CCK (excluding head) in the same species (Rojas-García et al., 2011) and in the present study, the increase in CCK in the study of Koven et al. (2002) is likely to be an increase in CCK in the brain and might act as a satiation signal instead of acting as a stimulation of pancreatic secretions. Similar conclusions were drawn for adult channel catfish (*Ictalurus punctatus*) and juvenile salmon (*Salmo salar*)(Peterson et al., 2012; Valen et al., 2011).
Finally, it has to be pointed out that standard length was chosen as the only proxy for growth due to several reasons. It reflects very well the developmental stage in larval cod (Finn et al., 2002) and is a conservative measure in starving larvae, which quickly lose protein and consequently body mass, which might have happened in the “control” group, but not in standard length. It has to be noted, that due to the analytical procedure, it was not feasible to assess body mass and/or protein content in individual larvae without any impact on the tryptic enzyme activity and CCK analytics. The focus on the analyses of tryptic enzyme activity and CCK in individual larvae in this study required to consider standard length as the reference.

5. Conclusions

Our study shows the individual development of CCK and the key enzyme trypsin in larval Atlantic cod and demonstrates a feedback mechanism among CCK and trypsin in regulating digestive processes in early larval stages of cod. The role of CCK as a trigger of pancreatic secretions is likely to mature as ontogeny proceeds, although spatial and temporal differences in CCK in different body compartments complicate the interpretation in developing fish larvae. Tryptic enzyme activity increased only slightly in early cod pointing towards limited digestive capacity early in development. Results of the 12 hour experiment revealed that tryptic enzyme activity increased immediately after a nutrient stimulus consisting of algae and rotifers, even with the administration of an algae solution without rotifers, which supports earlier findings that algae may play a key role in the maturation process of the digestive system in marine fish larvae. A second meal of rotifers the same day did not result in increasing enzyme activity, suggesting a limited proteolytic capacity in cod larvae to handle several meals in a short time period. Therefore, feeding times, frequency and amounts should be matched to the digestive capacity of the larvae to maximize nutrient utilization and growth. A reverse trend between CCK and tryptic enzyme activity was evident in all groups, indicating a negative feedback control in cod larvae similar to that found in mammals.

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Figure legends

Fig. 1. Standard length (mm) of Atlantic cod larvae during ontogenetic development 2 - 38 days post first-feeding (dpff). Data are presented as mean + SD (n = 10 - 15 at each sampling point).

Fig. 2. CCK content (fmol larva^{-1}) for length classes (standard length, 0.5 mm precision, 4.0 – 4.5, 4.5 – 5.0 etc.) of larval cod (excluding head). Larvae showing signs of the "Distended Gut Syndrome" (DGS) were excluded. Data are presented as mean + SD and numbers represent the number of larvae in each length class.

Fig. 3. Tryptic activity (hydrolysed substrate, nmol MCA min^{-1} larva^{-1}) for length classes (standard length, 0.5 mm precision, 4.0 – 4.5, 4.5 – 5.0 etc.) of larval cod (excluding head). Larvae showing signs of the "Distended Gut Syndrome" (DGS) were excluded. Data are presented as mean + SD and numbers represent the number of larvae in each length class.

Fig. 4. Relative gut fullness in the three feeding groups between 8:00 and 20:00. Algae were provided at 9:30 and 15:15 (all groups), rotifers at 10:30 ("one meal", "two meals) and 15:30 ("two meals"). Data are presented as mean + SD ("control" n = 5 individuals; "one meal", "two meals" n = 3 tanks). ⇩indicates the administration of algae, ⬇indicates the administration of rotifers. Different letters indicate significant differences between the three groups at a specific sampling point, "n.s." indicates non-significant differences (nested One-way-ANOVA, Student-Newman-Keuls test, $p < 0.05$). Values of gut fullness were transformed using the formula *gut fullness'=arcsin√gut fullness*.

Fig. 5. Daily pattern of CCK (fmol larva^{-1}) and tryptic enzyme activity (nmol MCA min^{-1} larva^{-1}) for cod larvae (excluding head) at 21 days post first-feeding: A = "control" (n = 5 individuals), B = "one meal" (n = 3 tanks), C = "two meals" (n = 3 tanks). Data are presented as mean +/- SD. ⇩indicates the administration of algae, ⬇ indicates the administration of rotifers. No significant differences in CCK and tryptic enzyme activity were found between all groups at each sampling point.

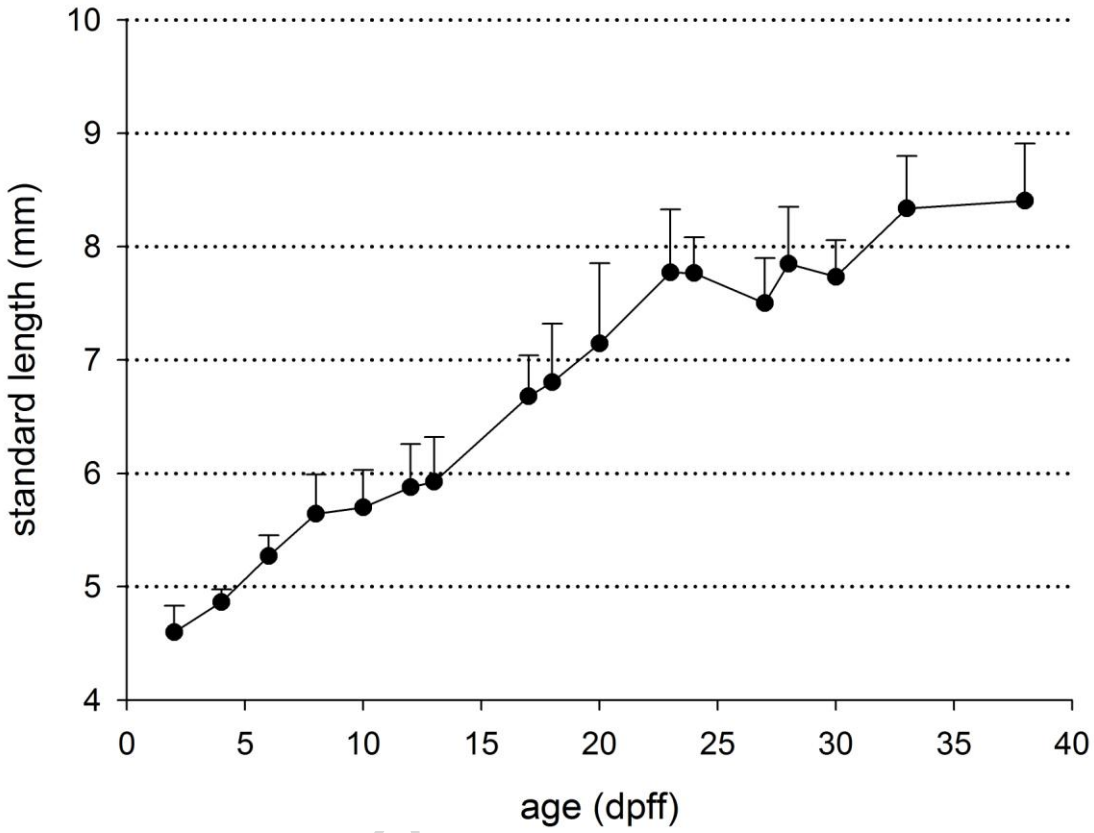

Fig. 1

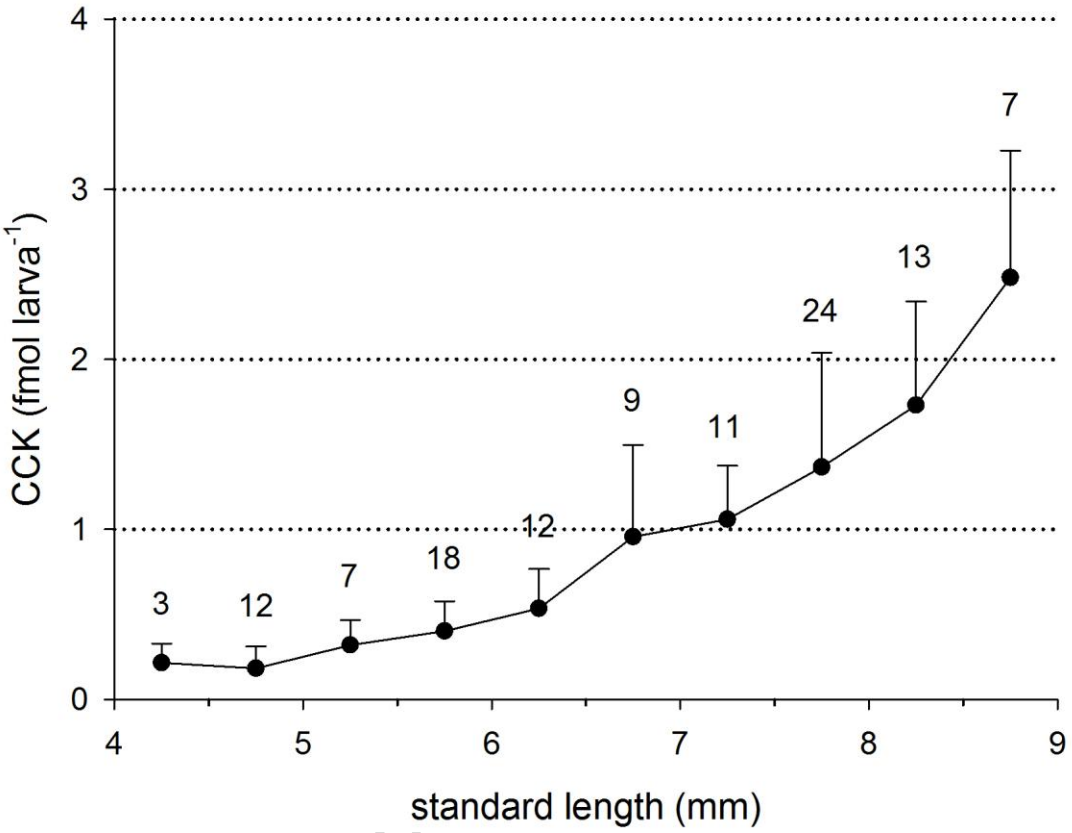

Fig. 2

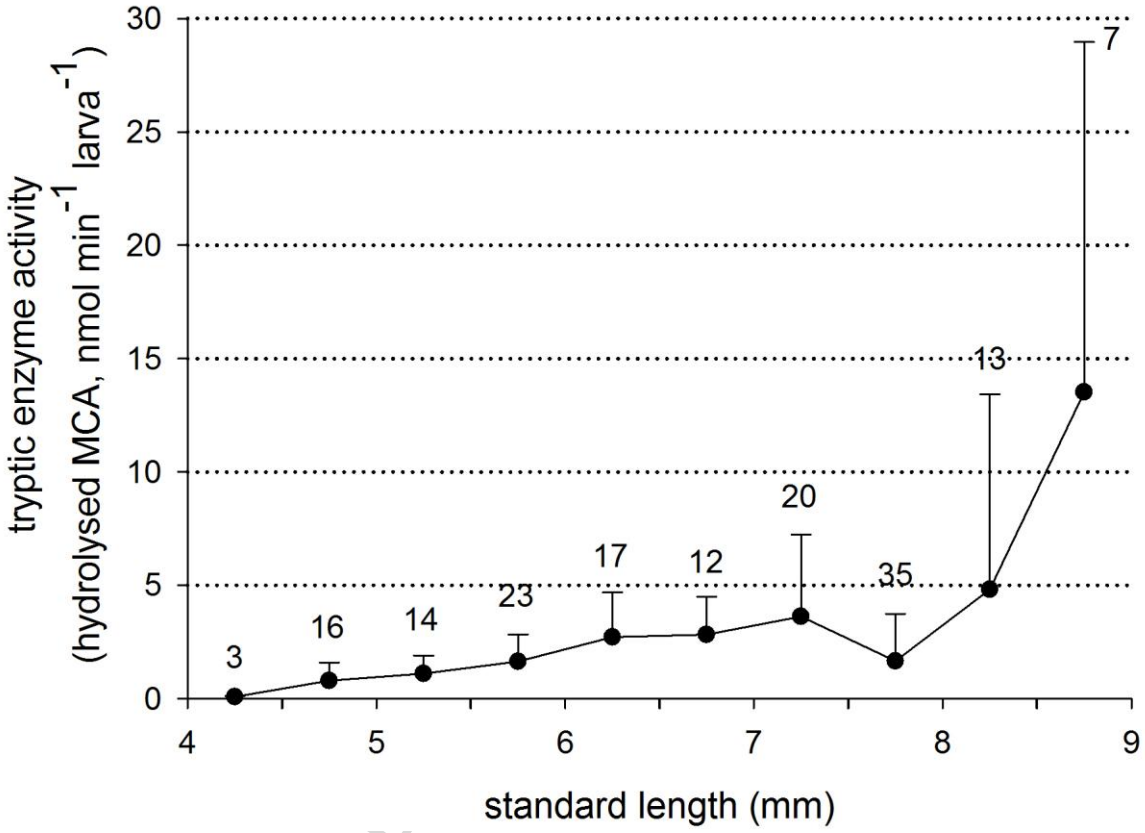

Fig. 3

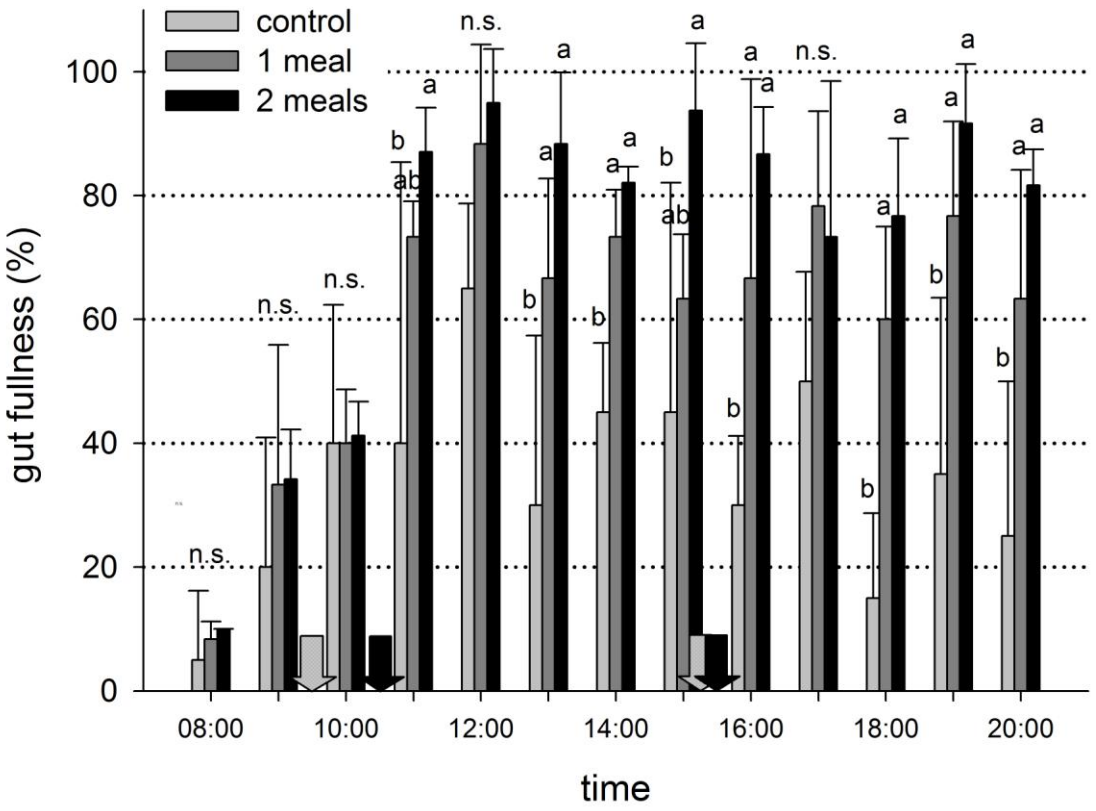

Fig. 4

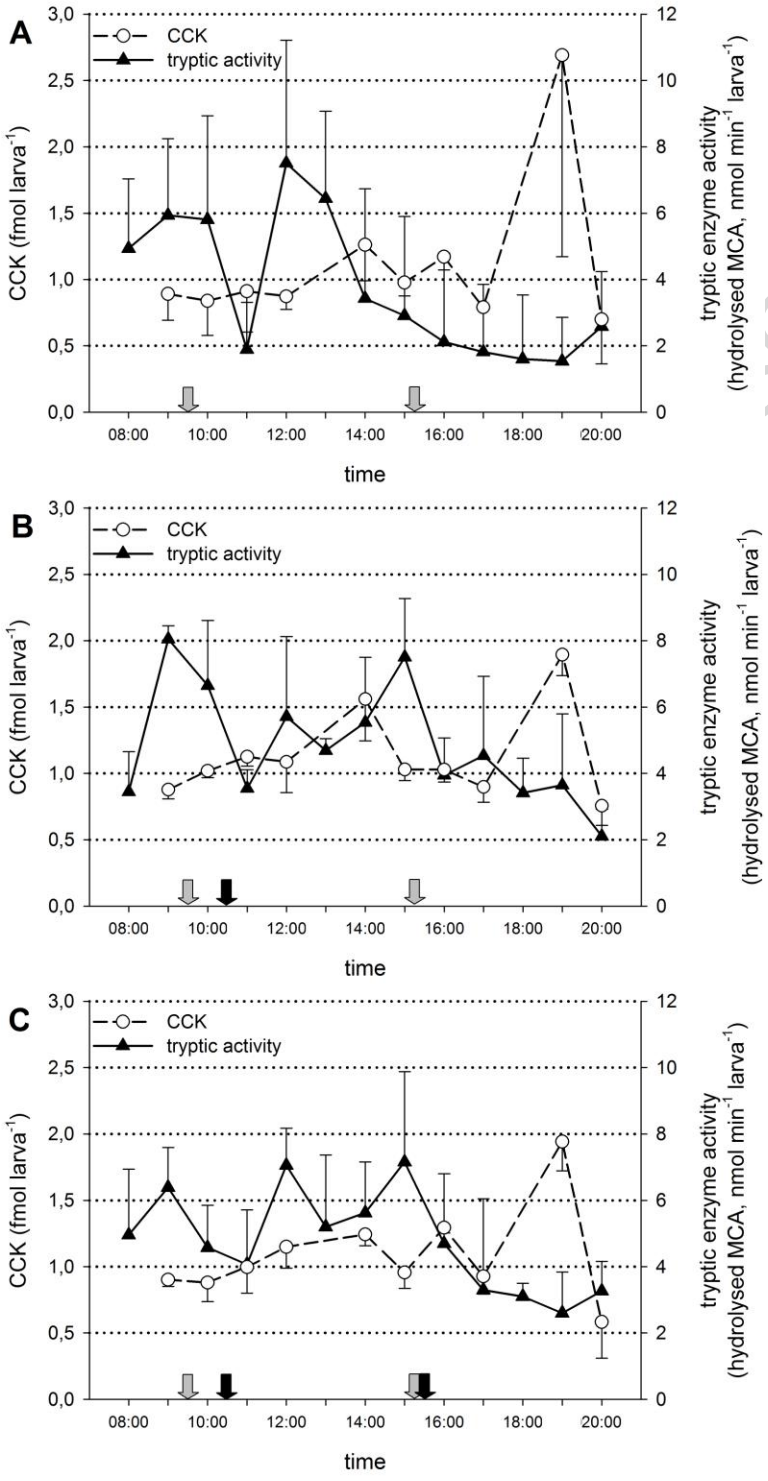

Fig. 5

Highlights

- We describe the ontogenetic development of CCK and tryptic enzyme activity in larval cod
- CCK is known to play a key role in regulating digestive processes
- CCK concentrations increased during ontogeny suggesting a growing role in regulating digestive processes
- A short-term experiment reveals a feedback mechanism between CCK and tryptic enzyme activity
- Cod larvae have limited regulatory and digestive capacity to handle several meals in a short period