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CCK concentration and tryptic activity
in early stages of finfish larvae

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Content

1 Introduction

In early life seafood was a privilege of coastal inhabitants only. In the present era of global trade it belongs to the commonly available goods, and its supply has formed a billion dollar industry. In 2002, world total fishery production (excluding aquatic plants) was reported to be 133 million tonnes, of which 93 million tonnes were landings from capture fisheries (Vannuccini, 2004). The question that concerns scientists who predict the total exhaustion of the oceans is: how can we enhance the recovery of endangered species and still satisfy the on-growing demand for marine products? Although sometimes described as an alternative to fisheries, aquaculture is still far from representing a solution to the decline of fish stocks. However, aquaculture contributes with a continuing growth to the world seafood production (Münkner and Kuhlmann, 2001; FAO, 2002) and will remain indispensable in the future. Thus, instead of totally condemning the production of farmed aquatic meat to co-responsibility and even amplification of over-fishing, enhanced interest should be concentrated in the development of sustainable culture practices and strategies that can still be profitable (Rosenthal, 1997; Burbridge *et al.*, 2001), a shift already being observed to become the key objective during the last years, although mainly in developed countries yet (FAO Fisheries Department, 2004).

Success in the cultivation of a target species begins with the reduction of costs in hatcheries. Apart from the availability of a broodstock able to reproduce successfully in captivity, which secures the independency from natural stocks, the reliable production of high quality fry with good growth and high survival rates is of considerable importance. During the first weeks after hatching, which include so-called "critical" periods for larval survival (Sifa and Mathias, 1987), marine fish larvae undergo drastic physiological and behavioural changes, like improvement of visual range and ability (Kvenseth *et al.*, 1996; Fiksen *et al.*, 1998; Carvalho *et al.*, 2004), increasing swimming activity (Skiftesvik, 1992) and the switch from endogenous to exogenous nutritional sources. These are again linked with a number of morphological changes. The rapid development requires a great deal of energy. Consequently, a nutritional supply of sufficient quantity and quality is essential for growth development and survival.

Natural prey organisms meet the nutritional demands of marine fish larvae the best (Evjemo *et al.*, 2003), but their use in commercial aquaculture restricts the production of fry to certain parts of the year and the fish farmers are not able to maintain control of prey species and size composition (Berg, 1997; Olsen *et al.*, 2000). A generalised feeding protocol for marine fish larvae begins with the administration of rotifers at the time of first feeding, followed by *Artemia* nauplii and larger *Artemia* stages as larvae increase in size. Formulated diets are then introduced and larvae are weaned from live feed organisms, a

process which varies in the time of start and duration, according to the species. Usually, micro-algae are additionally cultured as food for the zooplankton. Thus, most mariculture hatcheries culture at least three different live foods (micro-algae, rotifers, *Artemia*) to provide food for the larvae of a single target species (Southgate and Kolkovski, 2000 and 2005; Lee, 2003). The issues and problems associated with the use of live feed organisms are summarised in Table 1.

Table 1: Issues and problems associated with the use of live feed organisms in mariculture hatcheries (compiled from Sargent *et al.*, 1995; Evans, 2000; Southgate and Kolkovski, 2000 and 2005; Sorgeloos *et al.*, 2001)

Expense
Live food organisms contribute to 50-80% of hatchery operating costs. The price for *Artemia* cysts is subjected to high fluctuations depending on harvest success and quality

Facilities
Live food production requires substantial commitment of space and infrastructure

Nutritional inconsistency and/or deficiency
Live foods vary in their nutritional composition according to source, age and culture techniques. *Artemia* and rotifers represent only a suboptimal substitute for natural feeding organisms (e.g. copepodits), since they lack some essential nutrients - mainly highly unsaturated fatty acids - and must be enriched prior to use

Availability
Hatcheries rely on a continuing supply of adequate quantities of mostly imported *Artemia* cysts. In some countries there are also quarantine issues to be followed

Disease and/or crashes
The introduction of disease and live food culture crashes can be major problems for mariculture hatcheries

Recently, research has been conducted in developing systems and rearing protocols for intensive culture of copepods (Payne, 2000; ICES Mariculture Committee, 2004), as a way to overcome the necessity for using *Artemia.* However, most of the problems related to the use of live feed remain. The advantage of using formulated diets is not only economical but also nutritional. In contrast to live feed organisms, formulated feed particles can be adjusted in their size and nutritional composition according to the specific demands of the larval stages of a certain fish species.

The fish food industry as well as scientists dealing with the formulation of appropriate diets for larval stages have to consider a great number of factors influencing the acceptability and digestibility of the administered feed particles. These factors range from the

composition of macro- and micronutrients in particle sizes appropriate for the small opening of the larval mouth, to the performance of the feed particles in the water column, including stability and long lasting buoyancy, and characteristics like taste and colour (Dendrinos *et al.*, 1984; Bengtson, 1993; Rice *et al.*, 1994; Planas and Cunha, 1999; Cahu and Zambonino-Infante, 2001; D'Abramo, 2002; Cahu *et al.*, 2003; Langdon, 2003). The difficulty lies in achieving the optimum combination of all these factors, while also being adjusted to species-specific demands. Above all, the exceptional structure of the larval digestive tract - particularly of marine species (Kolkovski, 2001) -, which differs tremendously from that of adults, requires additional adaptations and in depth study, in order to understand the larval digestive physiology.

Pancreatic tissue has been identified histologically and/or histochemically in newly hatched larvae of several species (Beccaria *et al.*, 1991; Segner *et al.*, 1994; Kurokawa and Suzuki, 1996), and the importance of trypsin as the main proteolytic enzyme in the digestive tract of marine fish larvae that lack a morphological stomach (Govoni *et al.*, 1986), has also been recognised and described in numerous articles. Trypsin is produced by the pancreatic tissue as the inactive precursor trypsinogen (zymogen) and activated by the enzyme enteropeptidase. Further activation of trypsinogen occurs autocatalytic by trypsin itself. Storage of trypsinogen, as well as of other pancreatic zymogens occurs in the acinar cells, which - after stimulation - release their products into the gut lumen (Hjelmeland, 1995). However, the exact mechanisms of trypsinogen production and trypsin release are not yet fully known. Ueberschär (1993) demonstrated tryptic activity as the quantitatively most important proteolytic activity in larvae of herring and turbot. Based on results from different marine fish species, Ueberschär (2000) described 4 phases in the ontogenetic development of the larval tryptic activity, from hatching to metamorphosis (Fig. 1), and showed that the stage shortly after first feeding during which mass mortalities are often observed, is accompanied by low tryptic activity levels. Pedersen and Andersen (1992) found that trypsinogen secretion in herring larvae increases with prey size, even if non-biodegradable polysterene-latex spheres were offered instead of prey organisms, and thus suggested a neural initiation of trypsinogen secretion. Koven *et al.* (2002) proved the chemically mediated control of trypsin secretion by measuring higher trypsin activities in herring larvae after administration of bovine serum albumin in combination with specific free amino acids, rather than one of these components alone. Rojas-García and Rønnestad (2002) found a high correlation between tryptic activity and the hormone cholecystokinin in the gut of Atlantic halibut larvae, indicating that both factors contribute to the digestion process.
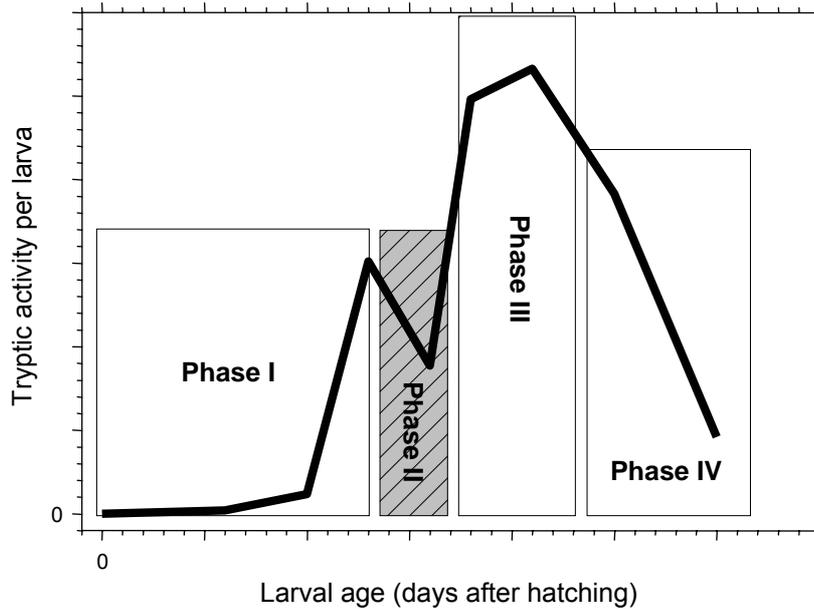
Fig. 1: 4-Phase hypothesis on the ontogenetic development of the tryptic activity in marine fish larvae from hatching to metamorphosis. Phase I: yolk-sac stage and initiation of exogenous feeding; Phase II: "critical" larval stage with decreasing tryptic activities, poor growth and high mortality rates; Phase III: sufficient production of trypsin (at optimal food supply), high growth rates; Phase IV: beginning of metamorphosis, during which tryptic activity is being partly replaced by the activity of pepsin in the developing stomach (after Ueberschär, 2000)

The gastrointestinal hormone cholecystokinin (CCK) has also a long research history. CCK was discovered by Ivy and Oldberg already in 1928, but further efforts to study this hormone showed slow progress, mainly due to difficulties in developing purification techniques and reliable methods for its determination. The problems related to the development of quantification methods for CCK have been summarised by Jansen and Lamers (1983), Rehfeld (1984) and Liddle (1998). One of the main contributing factors was that initially developed radioimmunoassays were performed with antisera directed against the common COOH-terminal pentapeptide sequence of CCK and the gastrointestinal hormone gastrin (Barrington and Dockray, 1976; Rehfeld, 1998a), and exerted a high degree of cross-reactivity. During the 1980s, highly specific CCK antisera were raised, with negligible cross-reactivity to gastrin (Himeno *et al.*, 1983; Ohgo *et al.*, 1988; Rehfeld, 1998b). Liddle *et al.* (1984 and 1985) developed also a bioassay based on the ability of CCK to stimulate amylase release from isolated rat pancreatic acinar cells. Since methodology problems were overcome, extensive research has been performed, various molecular forms of CCK have been characterised, and the involvement of CCK in the control of food processing and satiety in the gut as well as in specific brain regions of mammals (reviewed e.g. by Silver and Morley, 1991; Reidelberger, 1994) has been demonstrated by integrated actions on gastric emptying (Debas *et al.*, 1975; Liddle *et al.*, 1986; French *et al.*, 1993), gallbladder contraction (Byrnes *et al.*, 1981; Liddle *et al.*, 1985)

and pancreatic enzyme secretion (Soudah *et al.,* 1992). In context with the pancreatic enzyme secretion a feedback mechanism was proven between CCK and trypsin release (Owyang *et al.,* 1986).

Finding CCK in specific brain parts controlling the process of food intake, and at high concentrations in the mucosa, confirmed the regulatory effect of this hormone on digestion and satiety also in fish (Vigna *et al.,* 1985; Jönsson *et al.,* 1987; Sankaran *et al.,* 1987; Himick *et al.,* 1993; Himick and Peter, 1994 and 1995; Peyon *et al.,* 1999; Jensen *et al.,* 2001; Lee *et al.,* 2004). Most of the main actions of CCK have been demonstrated *in vitro* or *in vivo* in adult fish (Fig. 2). Exogenous CCK induced gut smooth muscle activity in cod (Jönsson *et al.,* 1987); delayed gastric emptying in rainbow trout (Olsson *et al.,* 1999); activated the gallbladder in coho salmon (Vigna and Grobman, 1977), rainbow trout (Aldman and Holmgren, 1987; Aldman *et al.,* 1992; Aldman and Holmgren, 1995), bluegill, killifish and bowfin (Rajjo *et al.,* 1988); and stimulated the secretion of trypsin and chymotrypsin from the pancreas in Atlantic salmon (Einarsson and Davies, 1996; Einarsson *et al.,* 1997). Although fishes are an extremely diverse and complex group, representing more than half of all vertebrates and accordingly, they have a great variation latitude (NRC, 1987; Håstein *et al.,* 2005), studying CCK in fish is of additional interest, since it can reveal actions of earlier origin that are not detectable in evolutionary older mammal species: e.g. duodenal HCl stimulated CCK release in rainbow trout (Aldman *et al.,* 1992), rabbit and dog (Berry and Flower, 1971; Chen *et al.,* 1985), but not in the prairie dog (Grace *et al.,* 1987), suggesting that CCK secretion by duodenal acidification is a response developed early in evolutionary history, which the prairie dog may have secondarily lost (probably due to feeding habits) (Aldman *et al.,* 1992).

CCK producing cells in the digestive tract have been detected immunohistochemically also in larval stages of turbot (Reiencke *et al.,* 1997), Japanese flounder and Japanese eel (Kurokawa *et al.,* 2000 and 2004), Atlantic halibut, bluefin tuna, ayu and Atlantic herring (Kamisaka *et al.,* 2001, 2002, 2003 and 2005). Plantikow *et al.* (1993) measured CCK and tryptic activity in larval rainbow trout and found depots of both even before first feeding. However, knowledge on the role of CCK in fish larvae is rather in a basic research stage, and much remains to be discovered with respect to synthesis, release and actions (Rønnestad, 2002; Rønnestad *et al.,* 2003).
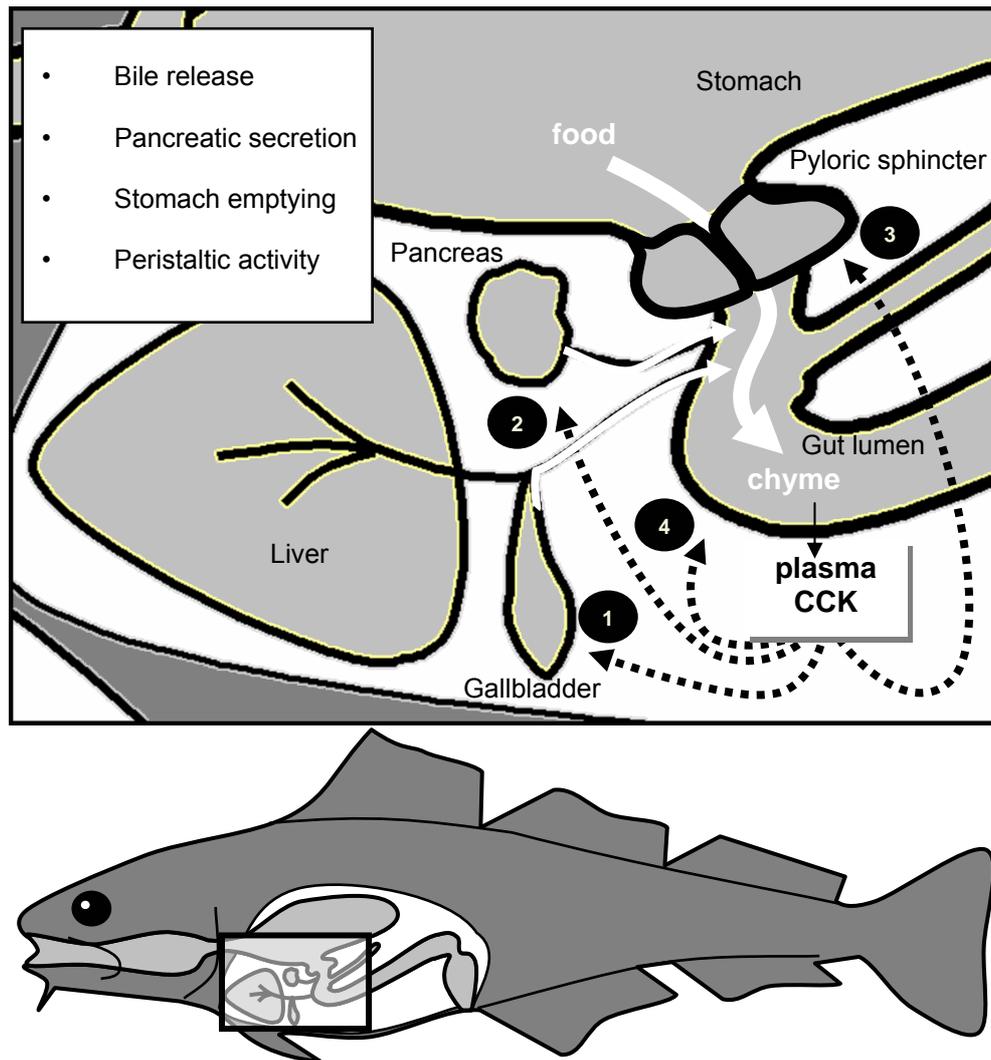
Fig. 2: Schematic presentation of the role of cholecystokinin in the control of digestive function in fish (after Rønnestad, 2002)

In relevance to the functional context of CCK and tryptic enzyme secretion, Herzig *et al.* (1997) and Rådberg *et al.* (2001) studied the effects of a plant protein called phytohemagglutinin (PHA): in rats, PHA significantly stimulated growth of the pancreas and the small intestine, stimulated CCK release *in vitro* and increased CCK plasma levels *in vivo*; in PHA treated piglets, a greater size of the pancreatic acinar cells and higher plasma levels of CCK were recorded, suggesting a contribution of PHA to the maturation of the pancreas.

PHA is a lectin extract from the red kidney bean *Vaseolus vulgaris*. Lectins are sugar-binding proteins of non-immune origin which agglutinate cells and/or precipitate glycoconjugates (Goldstein *et al.*, 1980; Dixon, 1981). Lectins have been found not only in plants but also in fish body fluids (Dash *et al.,* 1993), and play mainly a defensive role

against fungi, bacteria and predation (Alexander and Ingram, 1992; Arason, 1996; Ahmed, 2001). PHA comprises five isolectins. Each isolectin contains four subunits (building a tetramer held together by non-covalent forces), which are of two different types: leucocyte (L) and erythrocyte (E) reactive. L has high affinity to lymphocyte surface receptors and is responsible for the mitogenic properties of the isolectins. E is responsible for the erythrocyte agglutinating properties (Felsted *et al.*, 1977; Leavitt *et al.*, 1977; Hamelryck *et al.*, 1996). PHA is being routinely used *in vivo* and *in vitro* to initiate mitosis in cell cultures (Fan and Fox, 1990; Pendás *et al.*, 1993; Mizuno *et al.*, 1996; Otte *et al.*, 2001). The protein form of PHA prior to separation and purification of leucoagglutinin and erythroagglutinin is called PHA-P.

The aims of this work were to study the ontogenetic development of CCK in comparison to that of tryptic activity – which has been evaluated as a good indicator for even short-term changes in the nutritional condition of larval fish stages (Ueberschär and Clemmesen, 1992; Ueberschär *et al.*, 1992; Ueberschär, 1993) – in marine fish larvae. Furthermore, it was aimed to test the effects of externally applied CCK-8s (sulphated CCK-8, CCK molecule with 8 amino acid residues and a sulphate group in position 7 from the C-terminus) – which has been identified as the biologically most active endogenous CCK molecule (Aldman and Holmgren, 1987; Rajjo *et al.*, 1988) – on larval endogenous CCK and tryptic activity. For this purpose short-term and long-term applications were tested experimentally: a tube-feeding method was applied on larvae that were micro-injected *in vivo* with test solutions directly in the gut and sampled at different incubation periods after treatment, while immersion treatment was performed in bathing solutions of different concentrations repeatedly over a longer period. Since hormonal treatment doesn't find a wide acceptance by the consumers of farmed fish, additional to the external administration of the quite expensive pure physiological hormone, the plant protein phytohemagglutinin (PHA) was tested as a stimulant for CCK and consequent trypsin release.

Halibut, herring and cod were chosen for this study mainly because of their morphological differences (larval size and gut shape) at the onset of exogenous feeding. Much of the development which takes place during the embryonic phase in herring and cod, occurs during the yolk-sac phase in halibut (Karlsen *et al.*, 1998). While mouth opening in herring and cod is being observed within a few days after hatching, halibut larvae show a slow development in the mouth formation (Sæle *et al.*, 2004), which seems to be capable of opening and closing by the third week after hatching (Pittman *et al.*, 1990a). Herring larvae are a good model species for the type of nutritional experiments performed here, due to their simple and strait gut tube, composed of a narrow foregut which is separated by the foregut sphincter from the midgut, followed by the rectal sphincter and the hindgut (Tytler and Blaxter, 1988; Koven *et al.*, 2002; Kamisaka *et al.*, 2005). The gut of cod larvae is

much shorter and consists of the foregut and the hindgut, which form two ellipsoidal sections separated by a sphincter (Tytler and Blaxter, 1988). The larvae of halibut represent the biggest individuals at hatching, and by the time of first feeding they possess a rotating gut, differentiated in foregut, midgut and hindgut, which are distinguished from each other by sphincter muscles (Pittman *et al.*, 1990a; Luizi *et al.*, 1999; Kamisaka *et al.*, 2001). In culture, all three species depend on live food until metamorphosis and thus, details on their digestive physiological demands are needed for the development of successful formulated micro-diets.

Halibut and cod have been identified to have considerable potential for intensive farming (Olsen, 1997). Reduction of the wild halibut (Stickney, 2000) and over-exploitation of the wild cod fishery (Walden, 2000) have served to strengthen their market value and thus interest in aquaculture of these species. Now, halibut is being produced commercially in Norway, Canada, Iceland and Scotland; although intensive farming is possible, the production of juveniles is still the bottleneck, and many of the remaining problems are due to an incomplete understanding of the biology of the fish (Pittman, 2000; Shields, 2001). Cod breeding programs were established recently e.g. in Norway (Gildberg, 2004) and commercial production has been enabled, but cod farming is still in its infancy, mainly because of problems in the production of sufficient numbers of juveniles (Kjørsvik, 2000; Brown *et al.*, 2003). The overall survival of halibut after metamorphosis and weaning is given in the range of 0-10% (Olsen, 1997), and of cod (weaning starts not before 35 dah) in the range of 5-25% (Walden, 2000). Thus, research is still essential to improve start-feeding and weaning protocols.

2 Materials and Methods

All animal procedures and handling were in compliance with the Guide for the Care and Use of Laboratory Animals (NRC, 1996).

2.1 Origin and rearing of larval batches

2.1.1 Atlantic halibut (Hippoglossus hippoglossus)

On the 12th of June, about 1330 halibut larvae (250.2 day degrees after hatching, °dah, at 6.5°C; 38 days after hatching, dah) from a captive brood-stock of Atlantic halibut (*Hippoglossus hippoglossus*), were transferred from a commercial farm near Bergen to the culture facilities of the High Technology Centre in Bergen, Norway.

According to information from the personnel of the farm, these larvae were from one female and one male, and hatched on the 5th of May (day 0) with a hatching rate of 75%. Transportation of the larvae occurred in plastic bags filled with seawater and air (1:1), on ice. After arrival at the culture facilities in Bergen, the larvae were left in the bags in a temperature controlled room to acclimate to ~10°C, and were then counted before their release in a cubic, green coloured tank with stagnant seawater. 26% of the larvae died during the transport.

A closer inspection of the remaining individuals revealed that more than 50% of the larvae had still yolk-sac, and that all yolk-sac larvae had macroscopically visible fixed open jaws, a characteristic of the "gaper" disease, which results into serious larval loses in commercial farming. The mouths of individuals with this jaw deformity can not be closed voluntarily or by manipulation with a forceps, and the larvae are unable to feed actively (Pittman *et al.*, 1990a); consequently, they die in about one week after total absorption of their yolk reserve. Since they mostly stay passive on the water surface and don't swim actively for food, yolk-sac absorption lasts longer than in healthy individuals (Prof. K.A. Pittman, personal communication). Considering also the fact that in commercial farms temperature is often measured only at single points that are not necessarily representative for the entire cylindroconical incubation silo, it can be concluded that the rest of healthy larvae without yolk-sac could have been about 50°d older and passed the point of no return, since they were not yet first fed. Thus, it was decided to use only the "gaper" larvae in a short experiment (until 48 dah), since they still had yolk-sac, which should provide a basic nutritional supply (according to Terjesen *et al.* (2000) at 6°C yolk absorption in normal individuals is complete at around 46 dah).

The halibut larvae were reared in an initial water volume of 450 l (~2 larvae/l), which was reduced to 300 l during the last 5 days of the experiment, since the number of larvae was also reduced due to mortalities and sampling. Water temperature was maintained at 9.8 ± 0.2°C (n = 7). Salinity ranged from 34.1 to 34.2 and O_2 was 96.8 ± 1.4% (n = 7). Water exchange occurred daily via siphoning and refilling (about 1/3 of the water volume was exchanged daily). An overflow pipe fixed in a bottom hole in the middle of the tank was covered with gauze (500 μm), to prevent loss of larvae. Aeration was placed in the middle of the tank, to create an upward flow departing at the surface (Harboe *et al.*, 1998).

First feeding took place on the same day the larvae were transferred to the rearing tank (38 dah). Feeding was accomplished once daily, between 13:30 and 15:30 h. For this purpose, wild zooplankton consisting mainly of nauplii of different species (not further identified), copepods and rotifers, was caught every second day and kept at 10°C. Additionally, the water in the rearing tank was "greened" with algae of the species *Isochrysis* sp. and *Rhodomonas* sp., cultured at 25°C. The density and composition of the feeding organisms was checked every day in both the zooplankton and the larval rearing tank, and was maintained in the halibut tank at about 2000 individuals/l. The two algal species were added at a ratio of 1:1.

2.1.2 *Atlantic herring (Clupea harengus)*

Ripe Atlantic herring, *Clupea harengus* (Norwegian spring spawning herring) were caught near Bømlå (a small island south of Bergen, Norway) and transported within 3-5 hours on ice to the University of Bergen (Department of Biology). The brood fish was sorted according to sex, and females were stripped in individual containers. Table 2 shows the length and weight of the females used in the fertilisation trials. The eggs of each female were allowed to adhere to transparent acetate plates and were then artificially fertilised with the milt of 2-3 males at 15:00 h on the 27th of March (wet fertilisation, as described by Høie *et al.*, 1999). Finally, the fertilised eggs were washed with pre-filtered (0.2 μm) seawater of 34.5 salinity and a temperature of 7°C. The total fertilisation rate was more than 90%.

Table 2: Standard length and wet weight of adult female Atlantic herring (*Clupea harengus*), used for egg stripping at the end of March

Female	Standard length [cm]	Wet weight [g]
1	34	368
2	37	470
3	31	261
4	29	246

The acetate plates carrying the adhering eggs were incubated vertically in static 5 l glass beakers (2 plates per beaker) in pre-filtered (0.2 μm) seawater of 34.5 salinity. The beakers were placed within a water bath in a temperature controlled culture room with a regulated photoperiod of 12 hours light - 12 hours dark. The room temperature was adjusted at 6°C; the temperature in the water bath was controlled through a thermostat (type: 02 PT 623, 3 x 400 Watt; HETO, BIRKERØD, Denmark). A pump attached to the water bath system ensured an evenly circulation of the temperature-adjusted water among the beakers. Aeration was supplied to each beaker by two airstones. Water exchange was performed once a day by transferring the acetate plates into clean beakers with temperature-adjusted seawater.

On April 15th some of the plates were transferred to the culture facilities of the High Technology Centre in Bergen, Norway. The plates were hung in two round black tanks with a water temperature of 5.8-5.9°C. Hatching occurred on the 17th of April (day 0). The number of larvae in each tank was estimated at 2000 individuals and the water volume ranged between 390 and 500 l. Within the next two days water temperature was raised and maintained at 10.15 ± 0.2°C (n = 58), for the duration of the presented experiments (Fig. 3). Salinity ranged between 34.1-34.3 and oxygen was 87.2 ± 4.8 % (8.2 ± 0.8 mg/l, n = 10), measured after the daily feeding time by a *WTW TetraCon® 325* (WTW LF 330, Weilheim, Germany) and a *WTW CellOx 325* (WTW Oxi 330, Weilheim, Germany) probe, respectively. The culture facilities of the High Technology Centre have their water intake at 90-100 m depth off the Bergen coast, providing seawater of constant quality. The larvae were reared in stagnant water, which was exchanged daily via siphoning. An overflow pipe was fixed through a bottom hole in the middle of each tank, adjusted in its length to the desired surface level, and covered with gauze on top to prevent loss of larvae. A semi-closed cover was placed on each tank as shown in Fig. 4, in order to minimise light reflections from the white walls on the water surface.
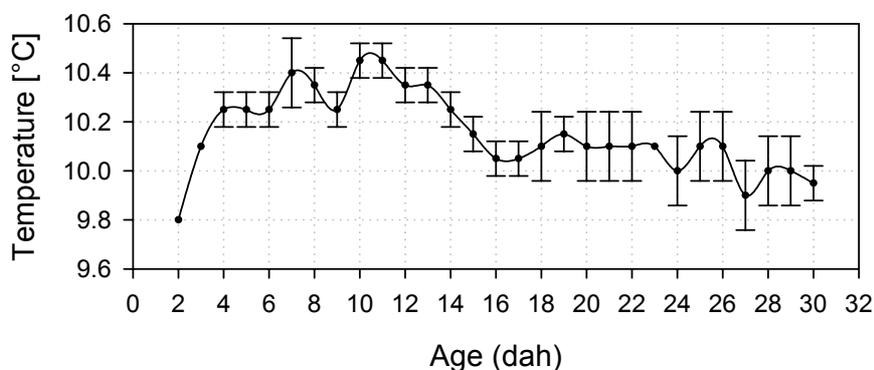

Fig. 3: Water temperature in the two rearing tanks of herring larvae from April 19th (2 dah) to May 17th (30 dah). Data points represent means ± S.D. of two measurements, one in each rearing tank

Between the 20th and 21st of April (3-4 dah) the mouth opening was observed. Consequently, first feeding occurred on the 20th of April (3 dah). Feeding was accomplished once daily, between 13:30 and 15:30 h. For this purpose, wild zooplankton (80-450 µm fraction) consisting mainly of nauplii of different species, copepods and rotifers, was caught every second day and kept at 10°C, in densities between 20 and 60 individuals/l. Additionally, the water in the rearing tanks was "greened" with algae of the species *Isochrysis* sp. and *Rhodomonas* sp., cultured at 25°C. The density of the feeding organisms in the herring tanks was maintained at about 2000 individuals/l. The two algal species were added at a ratio of 1:1.

The experiment was terminated at 30 dah.
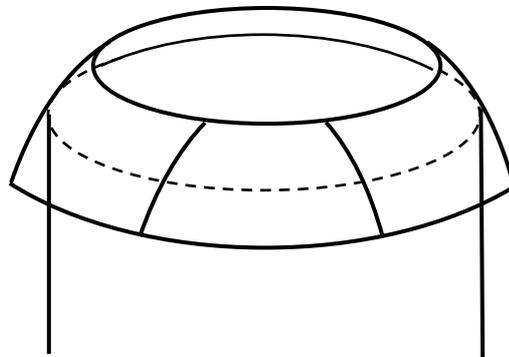
Fig. 4: Schematic drawing of the semi-closed cover placed on top of each rearing tank for herring larvae, in order to prevent light reflections from the white walls of the culture room on the water surface

2.1.3 *Baltic cod (Gadus morhua)*

Ripe Baltic cod (*Gadus morhua*) were caught on July 31st by trawling in the Bornholm Basin, central Baltic Sea, during a research trip with the R/V „Alkor" (Al 207/208). The eggs of one female were dry fertilised using the milt of five males. Table 3 shows the length and weight data of the adults used. The fertilised eggs were distributed to 500 ml plastic beakers containing 300 ml of pre-filtered (0.2 µm) seawater (17 beakers in total, mean number of eggs per beaker ± S.D.: 393 ± 180, density: 1-3 eggs per ml). Salinity was maintained at 14.5 and temperature at 7°C. Water exchange (at least ½ of the water volume) and removal of dead eggs occurred daily via siphoning. Until 8 days after fertilisation egg mortality was 43.8%.

Table 3: Length, wet weight of body and gonad, as well as maturity stage of adult Baltic cod (*Gadus morhua*), used for dry fertilisation at the end of July

Sex	Total length [cm]	Total wet weight [g]	Gonad wet weight [g]	Maturity stage
Female	49	1700	315	6
Male	39	493	32	6
Male	70	2460	287	6
Male	43	770	45	7
Male	44	910	80	6
Male	42	710	52	6

On the 8th of August the eggs were transferred to a temperature controlled culture room of the IFM-GEOMAR in Kiel (Department of Fishery Biology), Germany. On the 16th of August a hatching rate of 56.5% was recorded (day 0). About 6600 newly hatched larvae were transferred to two cubic plastic aquaria (8.25 l water volume, ~405 larvae/l), filled with seawater that had been pretreated with ozone (10 mg/h) and filtered through activated carbon. Aeration was supplied by airstones and water exchange via flow through system (40 ml/min). Light intensity was 200 lux, measured at the water surface. Table 4 shows the main water quality parameters in the rearing aquaria during the experimental period.

Table 4: Oxygen, temperature and salinity in the main rearing aquaria for cod larvae during the experimental period (16th-30th of August, 0-14 dah), given as means ± S.D. (n = 4 for oxygen and n = 7 for temperature and salinity measurements)

Aquarium	O_2 [%]	O_2 [mg/l]	Temperature [°C]	Salinity
1	98.8 ± 8.7	11.1 ± 0.85	6.2 ± 0.8	14.4 ± 0.3
2	99.4 ± 10.0	11.1 ± 1.1	6.3 ± 0.8	14.4 ± 0.3

Food was first offered on the 19th of August (3 dah) but was observed in the larval guts not before 5 dah. The administered feeding organisms were *Brachionus* sp. (culture density: 30-90 individuals/l, temperature: 29.2 ± 0.4°C and salinity: 15.9 ± 0.3, n = 5) fed with *Nanochloropsis* sp. (culture temperature: 30°C, salinity: 30), and were obtained from the Aquarium of the IFM-GEOMAR. 5-6 hours prior to feeding the zooplankton was enriched with essential highly unsaturated fatty acids (HUFA) by using *SUPER SELCO®* (INVE) (Table 5). The emulsion was prepared according to the instructions of the supplier (0.1 g/l for a rotifer density of 500 individuals/ml) and added to the well aerated rotifers in two portions. From day 13 after hatching onward, wild zooplankton caught in the Kiel fjord (fraction 150-300 µm) was additionally offered. The wild zooplankton consisted mainly of rotifers (*Synchaeta* sp.) and cladocerans (*Podon* sp.). The feeding organisms were offered once daily between 14:00 and 14:30 h, at a density of 2000 individuals/l until 4 dah and 4000 individuals/l from 5 dah to the end of the experiment.

Due to mass larval mortalities the experiment was terminated at 15 dah.

Table 5: Composition of the emulsion *SUPER SELCO*® used for nutritional enrichment of rotifers offered to cod larvae, as given by the supplier

SUPER SELCO® (INVE)	
Moisture (%)	30
Lipids (%)	65
Crude ash (%)	3
DHA/EPA ratio	1
Sum ω3 HUFA (mg/g dry weight)	400
Vitamin A (IU/kg dry weight)	1 500 000
Vitamin D3 (IU/kg dry weight)	150 000
Vitamin E (mg/kg dry weight)	3600
Vitamin C (mg/kg dry weight)	800

2.2 Micro-tube feeding method (short-term treatment)

The *in vivo* micro-tube feeding method used in this study was first described by Rust *et al.* (1993) and modified by Rønnestad *et al.* (2000a and b, 2001).

The equipment set-up for larval tube feeding via micro-injection is shown in Fig. 5. It consisted mainly of a microscope (WILD M3), under which the larva was positioned, and the micro-injector (Nanoliter 2000, WORLD PRECISION INSTRUMENTS) with the injection capillary (Fig. 6), which - filled with the test solution - was introduced into the larval gut. The capillary was secured into the micro-injector, washed with distilled water to remove any dust from the polishing step (described below, § 2.3.1), and then loaded with the test solution. The loading took longer, the higher the viscosity of the solution, and this was necessary in order to avoid introduction of air bubbles that can cause inaccurate injection volumes. After contamination with one test solution the used capillary was disposed. The applicable volume was adjustable in 4.6 nl intervals, up to a single injection volume of 69 nl. The actual volume used was 9.2 nl throughout the experimental period, but the concentration of the test solutions was adjusted to the wet body weight of the larvae, determined one day prior to injection performance. Since the aim of this study was to test the effects of the solutions only, no stimulation through the applicable volume and thus through the extension of the gut was desired. Consequently, based on pilot studies, the volume chosen was the smallest one that caused the slightest visible gut extension, in order to still enable a control of the micro-injection success. This visual control is only possible with larvae that have transparent gut areas. An alternative way to control the tube-feeding procedure, which also enables the monitoring of the gut passage, is to prepare coloured solutions (Rust *et al.*, 1993).
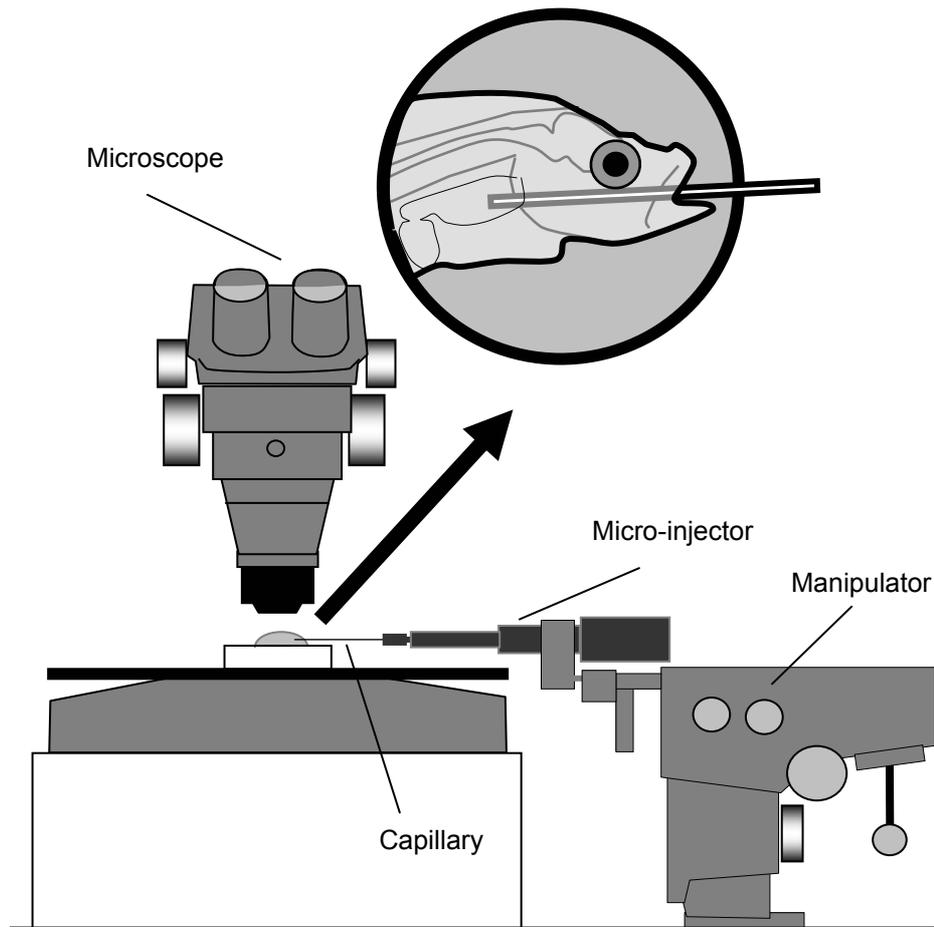
Fig. 5: Schematic drawing of the equipment set-up for tube-feeding of fish larvae via micro-injection (after Rønnestad, 2002)
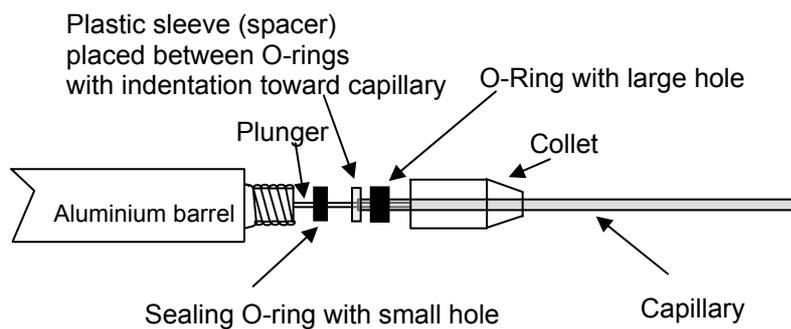

Fig. 6: Detailed configuration of the micro-injector components used for *in vivo* application of test solutions directly in the larval gut

Tube-feeding was performed on groups of randomly selected larvae, caught by a dip net from the rearing tanks early in the morning and kept in a 5 l bucket in the micro-injection

room, which was temperature controlled and adjusted equal to the rearing temperature (10°C) of the larvae treated. Larvae were removed individually from the bucket by a pipette with wide opening and anaesthetised in a Petri dish with 30 µg/ml MS-222 (Tricaine methanesulfonate, SIGMA-ALDRICH). Each larva was then gently placed in a droplet of seawater on the reverse side of a Petri dish under the microscope. Once the test solution was loaded and the larva positioned, the capillary was easily moved anterior of the larval mouth by the respective manipulator knobs of the micro-injector. The tip of the capillary was inserted through the mouth, past the oesophagus and into the foregut lumen. After application of 9.2 nl of the test solution with a single injection and a speed of 23 nl/sec, the capillary tip was slowly withdrawn. Fig. 7 documents the injection process on a halibut larva, but with a higher volume, in order to demonstrate the gut extension due to the micro-injected droplet.
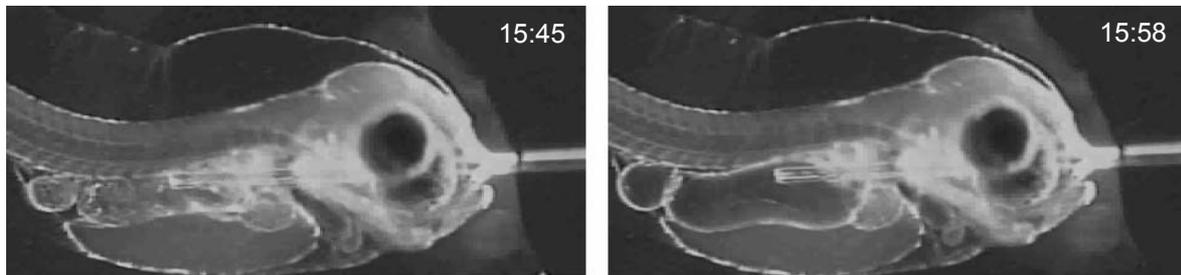

Fig. 7: Micro-tube feeding of a halibut larva. For demonstration purpose the micro-injection volume used here was higher than the actual one applied in the experiments (> 9.2 nl); (photos provided by Prof. I. Rønnestad, University of Bergen, Norway)

For each treatment and incubation period (specified below, § 2.3.2) at least 6 larvae were micro-injected. After injection, each larva was rinsed with clean seawater and transferred to an incubation well within a 6-well multidish (Cat. No. 150229, NUNC[TM] Brand Products, NALGE NUNC INTERNATIONAL), containing 5-6 ml of temperature-adjusted seawater. Opercular movements during rinsing effectively removed any contamination in the mouth and the branchial chambers (Rønnestad *et al.*, 2000a). The larvae remained in the incubation well until sampling (Fig. 8). Each larva was then gently lifted by the tail with a forceps, rapidly transferred to an Eppendorf tube with one droplet of seawater and flash frozen at -80°C, in order to interrupt any enzymatic activity. The frozen samples were transported on dry ice to the IFM-GEOMAR in Kiel, Germany and stored at -70°C until performance of biochemical analysis.
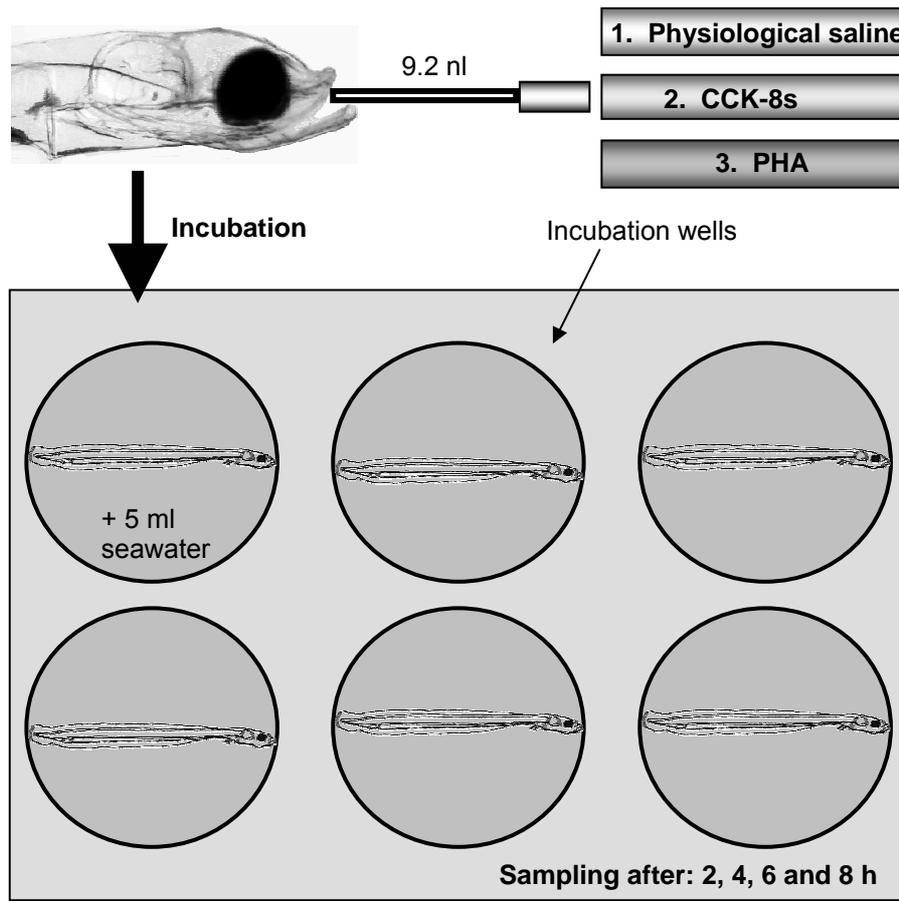
Fig. 8: Micro-injection groups and sampling times: each of the three test solutions was micro-injected to 24 larvae, 6 for each of the four incubation periods. Each test and sampling group was incubated in a separate 6-well multidish

2.2.1 Processing of the micro-injection capillaries

For the relatively big larvae of halibut, polycarbonate capillaries (T5026, SIGMA-ALDRICH) with an outer diameter of 0.19 mm were used (Fig. 9a). For larvae with a small mouth opening like herring and cod, the right tip size of the capillaries (outer diameter: 0.12-0.15 mm) (Fig. 9b) had to be formed by hand. For this purpose, the middle of a 67 mm glass capillary (Cat. No. 113.95-50, DRUMMOND SCIENTIFIC COMPANY) was constantly rotated with both hands over a Bunsen burner and pulled apart immediately when softened enough, in a way that allowed forming a 4-5 cm long tip (Fig. 10a). A small end of the new tip was broken and disposed, in order to ensure an unblocked opening. Finally, each hand-formed capillary tip had to be polished carefully to a conical shape, in order to round off the glass edges (Fig. 10b), which otherwise could damage the larval gut epithelium. A fine grade file leaf (grade 30 µm) attached to a drilling machine was the ideal solution for this step. The capillary tip was held quite upright over the rotating file leaf for about 5-10 minutes and checked under a microscope for remaining sharp edges.
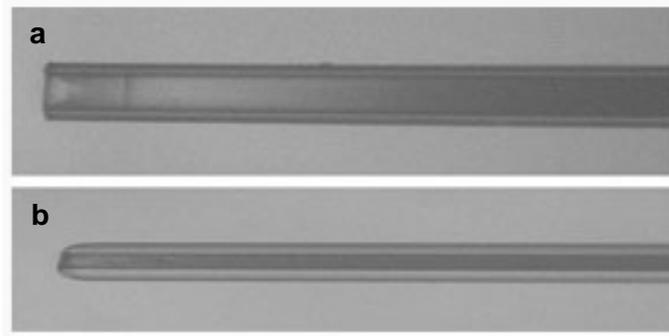
Fig. 9: Tips of the capillaries used for the micro-injections: a) plastic capillary tip with 0.19 mm diameter used for halibut larvae and b) hand polished glass capillary tip with 0.12-0.15 mm diameter used for herring and cod larvae (photos provided by Prof. I. Rønnestad, University of Bergen, Norway)
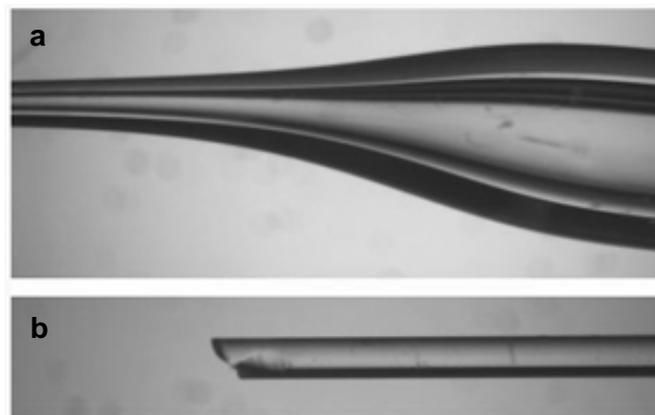
Fig. 10: Forming a glass capillary by hand: a) tapering to a tip with defined diameter after heating and b) unpolished tip

2.2.2 Tube feeding treatments and sampling groups (halibut and herring)

Micro-tube feeding was performed on halibut larvae at 40, 44 and 48 dah and on herring larvae at 5, 6, 11, 16, 20, 25 and 29 dah. Initial tube feeding tests with Atlantic cod larvae (10-12 dah reared at 10°C and 23 dah reared at 6°C) had to be abandoned because of very high post-treatment mortalities (50.8 and 76.4% respectively).

The micro-tube feeding series consisted of four groups of at least six larvae that were incubated for 2, 4, 6 and 8 h after being tube-fed a single injection (9.2 nl) of CCK-8s (cholecystokinin octapeptide sulphated, H-2080, MW = 1143.3, BACHEM), PHA-p (phytohemagglutinin, L-8754, SIGMA-ALDRICH) or physiological saline as a control. Physiological saline containing 0.9% NaCl (106404, M = 58.44, MERCK) and 1mM

$CaCl_2*2H_2O$ (SEC31307, M = 147.02, RIEDEL-DE HAËN) was also the carrier solution in which CCK-8s and PHA-p were dissolved. The incubation periods chosen were based on literature data (Rønnestad *et al.*, 2000b and 2001; Koven *et al.*, 2002), indicating that protein absorption from the gut to the body lumen takes up to 9 h (gut-clearance time). Only the 48 dah old halibut larvae were sampled at 0.5 and 1 hour after treatment.

Untreated larvae were sampled as an additional control at the same times as treated individuals, and also at non-treatment days once between 12:00 and 13:30 h.

The following starvation experiments were only performed with herring larvae kept in 5 l aerated buckets:
- Untreated larvae were deprived of food at 7, 15 and 24 dah for sampling over the following four days between 12:00 and 13:30 h.
- At the age of 8, 13 and 20 dah herring larvae were micro-injected with the three test solutions and kept under starvation conditions for sampling over the following 4 days in 24 h intervals.
- In order to test the mortalities due to handling after repeated micro-injections larvae aged 15 dah, which had been first injected and deprived of food two days before, were treated with a second injection and sampled 2 hours later.

Tables 6 and 7 show the detailed treatment and sampling schedules for halibut and herring larvae.

Table 6: Treatment and sampling schedule performed on halibut larvae: F = untreated; PS, PHA, CCK = injected with physiological saline, PHA or CCK-8s

Date	Injection day	Age (dah)	Sampling after treatment																						
			0.5 h				1 h				2 h				4 h				6 h				8 h			
05. Mai		hatching																							
12. Jun	first feeding																								
14. Jun	1	40									F	PS	PHA	CCK	F	PS	PHA	CCK	F	PS	PHA	CCK	F	PS	PHA	CCK
18. Jun	2	44									F	PS	PHA	CCK	F	PS	PHA	CCK	F	PS	PHA	CCK	F	PS	PHA	CCK
22. Jun	3	48		PS	PHA	CCK	F	PS	PHA	CCK																

Table 7: Treatment and sampling schedule performed on herring larvae: S = starving; F = untreated; PS, PHA, CCK = injected with physiological saline, PHA or CCK-8s; (PS, PHA, CCK) = starving after being injected once with the respective test solution; (PS-2, PHA-2, CCK-2) = starving after being injected twice with the respective test solution, on two consecutive days; X = no sampling possible due to mortalities

Date	Injection day	Age (dah)						Sampling after treatment															
								2 h				4 h				6 h				8 h			
17. Apr		hatching																					
20. Apr	first feeding	mouth opening																					
21. Apr																							
22. Apr	1	5					F	PS	PHA	CCK	F	PS	PHA	CCK	F	PS	PHA	CCK	F	PS	PHA	CCK	
23. Apr	2	6					F	PS	PHA	CCK	F	PS	PHA	CCK	F	PS	PHA	CCK	F	PS	PHA	CCK	
24. Apr		7					F																
25. Apr	(1)	8	S				F																
26. Apr		9	S	(PS)	(PHA)	(CCK)	F																
27. Apr		10	S	(PS)	(PHA)	(CCK)	F																
28. Apr	3	11	S	(PS)	(PHA)	(CCK)	F	PS	PHA	CCK	F	PS	PHA	CCK	F	PS	PHA	CCK	F	PS	PHA	CCK	
29. Apr		12		(PS)	(PHA)	X	F																
30. Apr	(2)	13					F																
01. Apr		14		(PS)	(PHA)	(CCK)	F																
02. Mai	(2-2)	15		(PS-2)	(PHA-2)	(CCK-2)	F																
03. Mai	4	16	S	X	X	X	F	PS	PHA	CCK	F	PS	PHA	CCK	F	PS	PHA	CCK	F	PS	PHA	CCK	
04. Mai		17	S	X	X	X	F																
05. Mai		18	S				F																
06. Mai		19	S				F																
07. Mai	(3), 5	20					F	PS	PHA	CCK	F	PS	PHA	CCK	F	PS	PHA	CCK	F	PS	PHA	CCK	
08. Mai		21		(PS)	(PHA)	(CCK)	F																
09. Mai		22		(PS)	(PHA)	(CCK)	F																
10. Mai		23		X	X	X	F																
11. Mai		24		X	X	X	F																
12. Mai	6	25	S				F	PS	PHA	CCK	F	PS	PHA	CCK	F	PS	PHA	CCK	F	PS	PHA	CCK	
13. Mai		26	S				F																
14. Mai		27	S				F																
15. Mai		28	S				F																
16. Mai	7	29					F	PS	PHA	CCK	F	PS	PHA	CCK	F	PS	PHA	CCK	F	PS	PHA	CCK	

One day prior to micro-injection performance ~10 larvae were individually weighed on a balance (METTLER AT 261 Delta Range®, 0.00 mg) in order to adjust the concentration of the respective test solution to the larval wet body weight. Table 8 shows the wet weights of halibut and herring larvae that were used for the calculation of the appropriate concentrations.

Table 8: Mean wet body weights ± S.D. (n = 7-10) of halibut and herring larvae used as references for the preparation of the test solutions prior to micro-tube feeding (concentration adjustment)

Species	Age (dah)	Wet weight [mg]
Halibut	39	6.785 ± 0.26
	43	6.93 ± 1.74
	47	5.5 ± 0.14
Herring	4	1.5
	7	1.76 ± 0.19
	10	2.43 ± 0.7
	12	2.63 ± 0.53
	15	3.87 ± 0.96
	19	5.24 ± 1.2
	24	9.14 ± 3.19
	28	14.68 ± 4.01

Concentrations used for the preparation of the test solutions were based on literature data.

At the time of experimental planning, the only available reference about externally administered CCK in fish of stages earlier than adults and its effect on trypsin was that of Einarsson *et al.* (1997). In that study, a porcine CCK extract (P4429, SIGMA-ALDRICH) was used for intraperitoneal injections in sibling Atlantic salmon (13-35 g wet weight), and produced a dose-dependent release of trypsin. The difficulty was that the authors gave the used concentrations in "Crick Harper Raper units", which were used until the late 1960s to express the activity of pancreozymin. After purification by Jorpes and Mutt (1966), who showed that both pancreozymin and CCK are one and the same hormone, the activity expression in "Crick units" was proved to be unsuitable (Bodanszky *et al.,* 1973). Today, it is difficult to reconstruct studies that express concentrations in Crick units, particularly because different purifications result in different activities, and this information is not provided in a standardised unit by the different suppliers. However, according to the supplier's (SIGMA-ALDRICH) information that 1 mg solid porcine CCK-pankreozymin extract has an activity of 4-6 Crick units, the amounts weighed by Einarsson *et al.* (1997) were calculated in the range between 0.17 ng and 0.125 µg per mg wet weight. For the present experiments it was decided to refer to the minimum concentration used by Einarsson *et al.* (1997) and point out that the actual activity is expected to be higher, since purified CCK-8s

was used here. The applied CCK-8s concentration on halibut and herring larvae was 0.1143 ng per mg wet weight (equivalent to 100 fmol per mg wet weight).

In a previous study (Drossou *et al.*, 2006) effects on CCK level and tryptic activity of Nile tilapia larvae were observed when PHA was dry mixed with a commercial feed, in a concentration of 0.05% of the larval wet body weight. In contrast to that application type, the micro-tube feeding method ensures the direct application of the whole concentration into the larval gut and excludes higher losses in the surrounding medium (seawater in the rearing tank). Thus, and because of the lack of further studies with PHA on fish larvae, the PHA concentration used here was chosen according to a study of Pendás *et al.* (1993), who injected Atlantic salmon (10-20 g body weight) with 0.003% PHA of wet body weight, and obtained responses to the treatment from all animals. Although the aim of the authors was to produce higher cell metaphase yields and was not directed to effects on CCK or tryptic activity, the PHA concentration administered aught to represent a practically evaluated dose for basic research experiments.

2.3 Immersion treatment (long-term) and sampling groups (cod)

Mangor-Jensen and Adoff (1987) and Tytler and Blaxter (1988) first reported active drinking in early larval stages of cod larvae, even in near iso-osmotic water with a salinity of 16. This osmoregulatory mechanism was used in the present experiment, during which cod larvae were exposed to CCK-8s in two constant concentrations via long-term immersion treatment: 1 µg of CCK-8s in 40 ml seawater (0.025 pg/nl, equivalent to 0.022 µM) and 10 µg CCK-8s in 40 ml seawater (0.25 pg/nl, equivalent to 0.22 µM).

Since the concentration in each of the two solutions was the same during the whole experimental period, the daily uptake by the larvae varied with the drinking rate at the respective developmental stage. The drinking rate of cod larvae was not determined in the present experiment and thus, the resulting concentration of the external CCK-8s in the larval gut can not be exactly estimated. Brown and Tytler (1993) determined that the water drunk by turbot larvae was absorbed prior to its passage into the rectum at a rate of 65-74%, when larval rearing occurred in full strength seawater, and at 30-35% in 50%-seawater. Beside water parameters, the larval drinking activity – which varies among species – is being positively influenced by body size and feeding activity (Tytler *et al.*, 1990). Additionally, it must be noted that different methods for the determination of larval drinking rates may have been the reason for varying results among studies. By using different labelling methods for the applied solutions, Mangror-Jensen and Adoff (1987) determined an individual drinking rate of about 3 nl/h, for cod larvae aged 3-7 dah and reared at 5°C and a salinity of 34. In contrast, Tytler and Blaxter (1988) came to 7.5 nl/h

with cod larvae aged 7 dah and reared at 7.5°C and a salinity of 32. The authors also noted that drinking rates in seawater with a salinity of 32 are approximately double those in seawater with a salinity of 16. Considering the results obtained by Tytler and Blaxter (1988), only an approximate estimation of the CCK-8s concentrations administered in the present study can be made: with an individual drinking rate of approximately 3-4 nl/h (at a rearing salinity of 14), the intaken CCK-8s concentration was estimated in the range of 0.075-0.1 pg/larva and of 0.75-1 pg/larva after immersion in the low and the high concentrated solution, respectively.

At 4 dah 580 larvae were randomly selected from the two cod rearing aquaria and distributed to four plastic aquaria (two aquaria per treatment). Each aquarium contained 400 ml seawater and was aerated through one airstone. Abiotic conditions and feeding regime were the same as for the controls. From 4 to 14 dah the larvae of each treatment group were immersed daily between 10:00 and 11:00 h in 40 ml of the respective CCK-8s test solution (0.02 µM or 0.2 µM). After 1.5 hours 6 larvae were randomly sampled from each treatment group, while the remaining larvae were transferred to a glass with clean seawater and then back to their rearing aquarium.

Untreated larvae were sampled as a control at the same time as the treated individuals.

Before first feeding, 50 larvae were removed from the control group and kept without food in a plastic aquarium with 400 ml seawater and aeration, for sampling over the following days.

2.4   Brief overview of treatment conditions and experimental groups

Halibut larvae
First feeding at 38 dah (250°dah).
Short-term micro-tube feeding treatment at 40, 44 and 48 dah.
Experimental groups:
- "Gaper" (regularly fed but due to jaw deformity not actively feeding), untreated yolk-sac larvae: untreated control.
- "Gaper" (regularly fed but due to jaw deformity not actively feeding), yolk-sac larvae injected with physiological saline (PS): treated control.
- "Gaper" (regularly fed but due to jaw deformity not actively feeding), yolk-sac larvae injected with CCK-8s or PHA solution.

Analysis of whole larvae (body and head).

Herring larvae

First feeding at 3 dah.

Short-term micro-tube feeding treatment at 5, 6, 11, 16, 20, 25 and 29 dah.

Experimental groups:

- Regularly fed, untreated larvae: untreated control.
- Regularly fed larvae injected with physiological saline (PS): treated control
- Regularly fed larvae injected with CCK-8s or PHA solution.
- Starving, untreated larvae.
- Starving larvae injected with PS, CCK-8s or PHA solution once.
- Starving larvae injected with PS, CCK-8s or PHA solution twice.

Analysis of larval bodies only (without head).

Cod larvae

First feeding at 3 dah.

Long-term immersion treatment from 4 to 14 dah.

Experimental groups:

- Untreated eggs.
- Regularly fed, untreated yolk-sac larvae: untreated control.
- Regularly fed yolk-sac larvae immersed in 0.02 or 0.2 μM CCK-8s solution.
- Unfed, untreated yolk-sac larvae.

Analysis of whole larvae (body and head).

2.5   Combined analysis of CCK concentration and tryptic activity

In order to determine the CCK concentration and the tryptic activity in individual fish larvae a combination of two methods was applied. CCK levels were assayed by a radioimmunoassay (RIA) kit (Cat. No. RB 302, EURIA-CCK, EURO-DIAGNOSTICA AB). Tryptic enzyme activity was measured by a fluorometric method as described by Ueberschär (1988 and 2000). The protocol for the preparation of each larval sample prior to use in both analyses was described by Rojas-García *et al.* (2001) and was performed here with some modifications (Fig. 11).
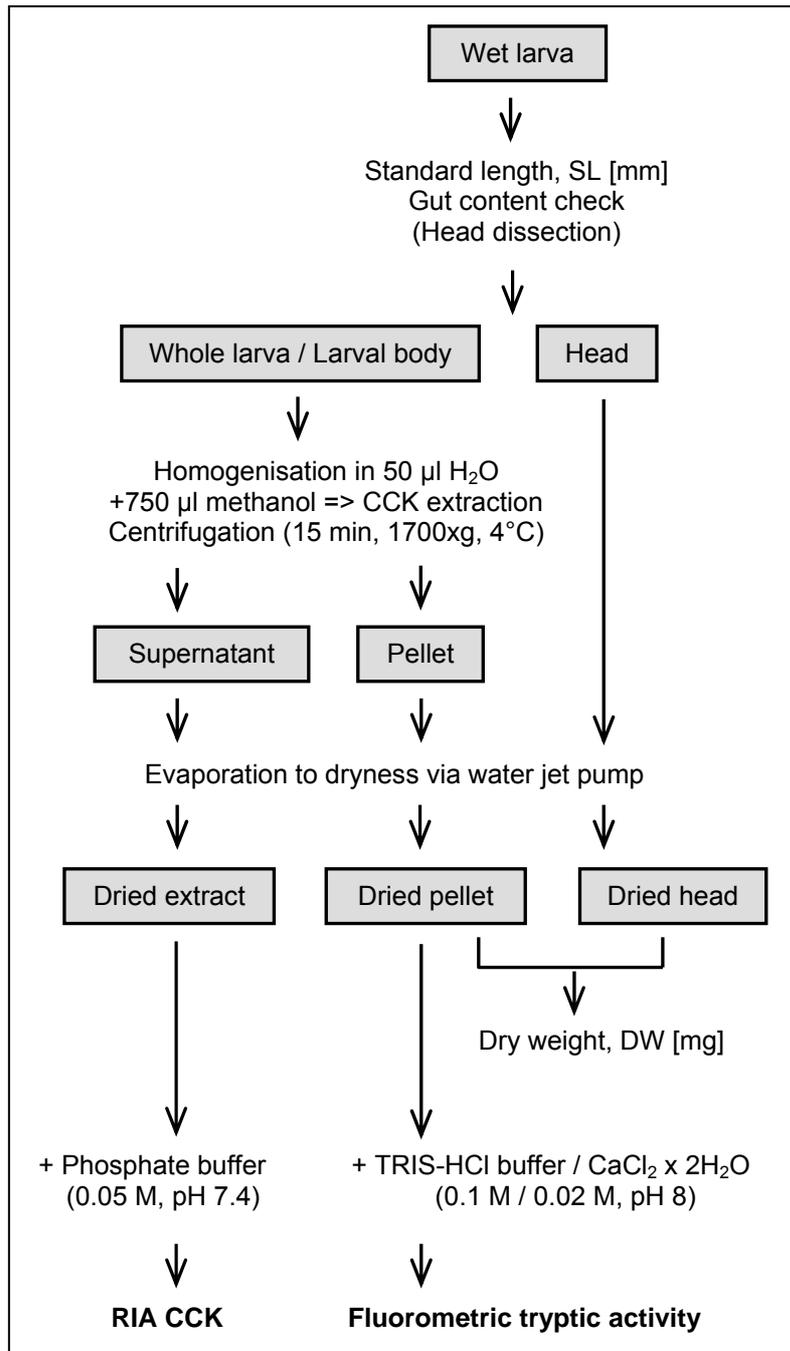
Fig. 11: Flowchart of the preparation protocol for individual larval samples prior to their use in two analytical methods for CCK content and tryptic activity

2.5.1 Preparation of individual larval samples

Frozen samples were placed in a plastic tub with ice and allowed to thaw slowly at 0°C. Each larva was rinsed with distilled water, checked under the microscope (WILD, M5A) for gut completeness, and the standard length (SL) was measured. Only larvae with completely intact guts were processed. While halibut and cod larvae were processed in whole, the head of herring larvae was dissected out, in order to exclude brain CCK concentrations (Rojas-García *et al.*, 2001). Larval head dissection was performed inside an Eppendorf tube by positioning the larva near the mouth of the tube and cutting with a scalpel in a way that allowed gut contents to remain intact (MacKenzie *et al.*, 1999).

Each larva, larval body and head was transferred to an individual tared Eppendorf tube. Whole larvae or larval bodies were homogenised in 50 µl distilled water with a fitted motorised pestle (Pellet Pestle® Motor, KONTES GLASS COMPANY). For extraction of CCK 750 µl of 100% methanol were added, and the samples were vortex-mixed for 30 s and left for 30 min in the tub with ice. The tubes were centrifuged for 15 min (1700 x g, 4°C), and then each sample was split in two by carefully transferring the supernatant to a clean Eppendorf tube. Tubes with pellets (methanol-insoluble fish precipitate) and supernatants (CCK methanol extract) as well as those containing the dissected heads were evaporated to dryness in a vacuum desiccator attached to a water-jet pump. The dried pellets and heads were used to determine the larval dry weight with a microbalance (METTLER AT261 delta range®). Thereafter the heads were disposed, and the pellets and CCK extracts were stored at -20°C until further processing for analysis of tryptic activity and CCK concentration, respectively.

2.5.2 CCK radioimmunoassay

All the steps of the radioimmunoassay were performed in the Isotope Laboratory of the IFM-GEOMAR under consideration of the regulations for proper handling of radioactive material.

The CCK concentration in the extracts was assayed by a competitive radioimmunoassay (RIA) using a rabbit antiserum raised against sulphated CCK-8 (CCK-8s), and the tracer ^{125}I-CCK-8s. CCK-8s in standards and samples competes with the ^{125}I-CCK-8s in binding to the antibodies: ^{125}I-CCK-8s binds in a reverse proportion to the concentration of CCK-8s in standards and samples. Antibody-bound ^{125}I-CCK-8s was separated from the unbound fraction using double antibody solid phase (anti-rabbit-Ig). The radioactivity of the bound fraction was measured in a gamma counter (BF 5000 G1, BERTHOLD), and the CCK content was interpolated from the standard curve, which was produced on each day of RIA

performance. Table 9 summarises the characteristics of the assay, as given by the supplier of the RIA-kit.

Table 9: Characteristics of the RIA-CCK assay, as given by the supplier (EURO-DIAGNOSTICA AB)

Sensitivity		
The lowest detectable concentration is 0.3 pmol/l		
Accuracy		
A mean recovery of 80% was obtained when known amounts of CCK-8s were added to samples		
Precision		
Intra assay variation:	Level (pmol/l)	Coefficient of variation (CV)
	4.4	5.5 %
	20.6	2.0 %
Inter assay variation:	4.2	13.7 %
	20.6	4.1 %
Specificity		
The following cross-reactions have been found:	Peptide	Cross-reaction
	CCK 26-33 sulphated	100 %
	CCK 26-33 non sulphated	< 0.01%
	CCK 30-33	< 0.01%
	Gastrin 17 sulphated	0.5 %
	Gastrin 17 non sulphated	< 0.01%

Prior to use the RIA-kit reagents were brought to room temperature and reconstituted as specified by the supplier with deionised double-distilled water. CCK-8s working standards with 7 different concentrations (0, 0.78, 1.56, 3.12, 6.25, 12.5 and 25 pmol) were prepared by dilution of a 50 pmol CCK-8s standard with 0.05 M phosphate buffer (diluent, pH 7.4).

The dried CCK extracts from herring larval bodies and halibut or cod whole larvae were solubilised in 500 µl diluent (factor diluent = 0.5), vortex-mixed and left on the bench for 30 min before used in the RIA procedure. 200 µl of sample or standard (standards were performed in duplicates) were pipetted in an Eppendorf tube (factor sample = 1) together with 500 µl of anti-CCK-8s, vortex-mixed and incubated for 44-50 h at 2-8°C in the dark. For the determination of the non-specific binding (NSB), 700 µl diluent were pipetted in a tube (in duplicate) without antiserum; further handling was the same as for samples and standards. After 2 days 500 µl of ^{125}I-CCK-8s were added to the tubes, vortex-mixed and incubated for another 92-100 h at 2-8°C in the dark. After 4 days 100 µl of double antibody solid phase was added to the tubes. This reagent was stirred continuously with a magnetic stirrer during pipetting. The tubes were incubated for final 30-60 min at 2-8°C in the dark and centrifuged for 15 min (1700 x g, 4°C). The supernatants were removed carefully with

a pipette tip attached to a water jet pump, and the radioactivity in the pellets was counted in a gamma counter for 10 min (counts per minute were obtained, cpm).

As a control, the total counts (TOT) were determined in 500 µl of ^{125}I-CCK-8s. The counts obtained were adjusted to the radioactive decay after the following formula:

$$A_t = A_0 * e^{-(\ln2 * t)/60}$$

A_t = radioactivity on the day analysis was performed

A_0 = radioactivity (cpm of the TOT) given by the supplier

t = number of days from the time the radioactivity was measured by the supplier (activity reference date given by the supplier) until the day of analysis performance

60 = half-life of the iodine isotope

Additional controls were performed through calculation of the maximum binding (B_0 / TOT * 100, B_0 = counts of the zero-standard) and the non specific binding (NSB / TOT * 100), which were always within the limits given by the supplier.

From the counts obtained for samples, standards and TOTs, the NSB counts were subtracted. The CCK concentrations were calculated as pmol/l by interpolation from the standard curve, produced by plotting the bound fractions (B / TOT, B = counts of the respective standard) versus the concentrations of the CCK-8s standards (Fig. 12). The sample CCK concentrations were expressed in fmol/larva.
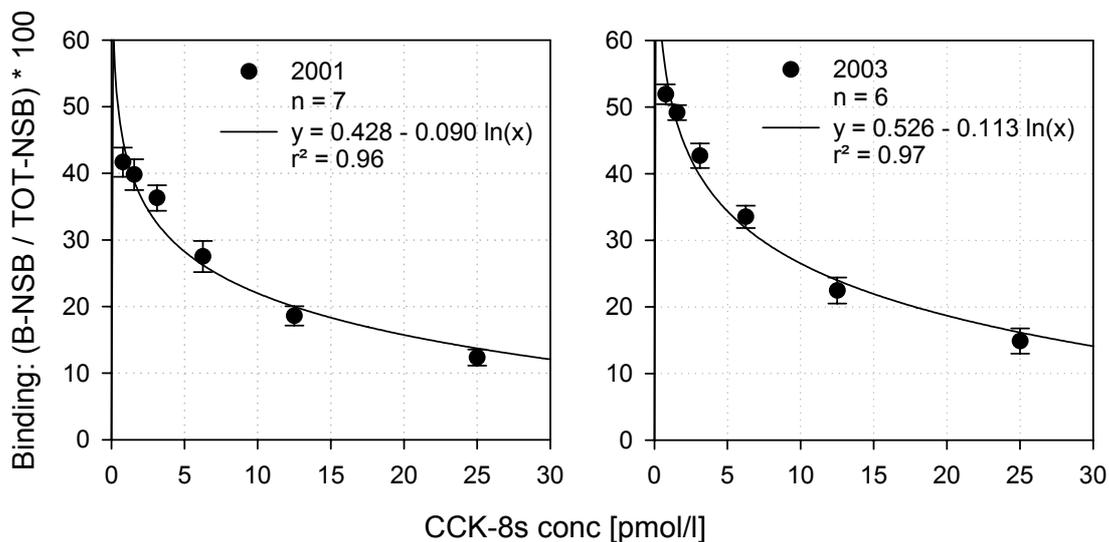
Fig. 12: Means ± S.D. of standard curves (logarithmic function analysis, p<0.005) produced on different days of RIA performance in 2001 and 2003, from which the CCK concentrations of samples were calculated. The curves produced in one year were performed with reagents from RIA-kits of the same serial number. (B = counts of standard, NSB = counts of non-specific binding sample, TOT = total counts of the ^{125}I-CCK-8s)

In order to evaluate the accuracy of the equipment used in the IFM-GEOMAR, a series of standards and samples was additionally measured with a second gamma counter (LB MAG 315R, BERTHOLD) at the Central Isotope Laboratory of the Biological Department of the University in Kiel, Germany. The measurements showed a very good reproducibility of 99.6% for standards and 95.75% for samples (Fig. 13 and 14).
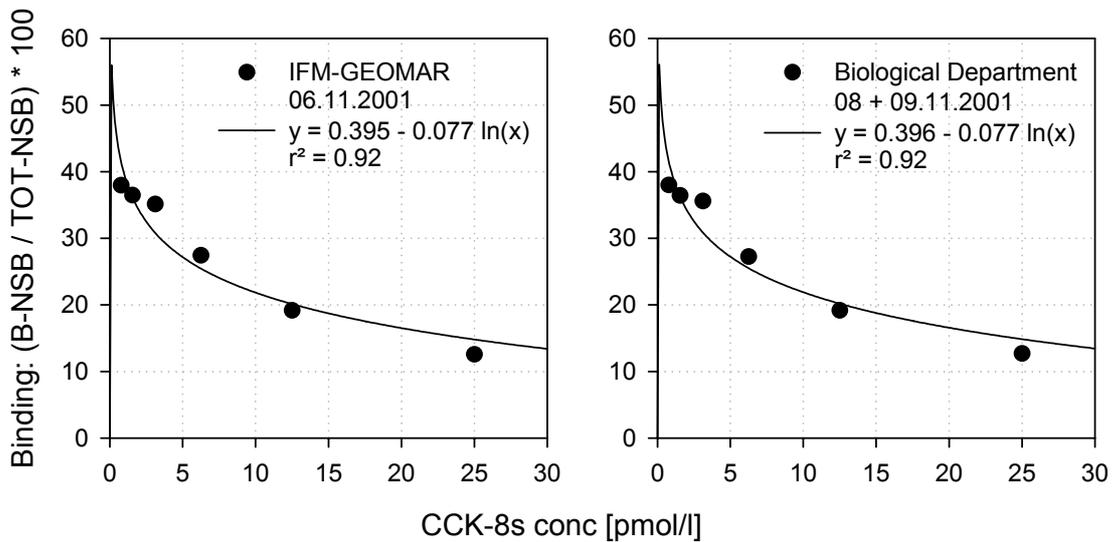
Fig. 13: Comparison of standard curves (logarithmic function analysis, $p<0.0005$) for the calculation of CCK concentrations, produced by counts obtained from two different gamma counters available in two different isotope laboratories. (B = counts of standard, NSB = counts of non-specific binding sample, TOT = total counts of the ^{125}I-CCK-8s)
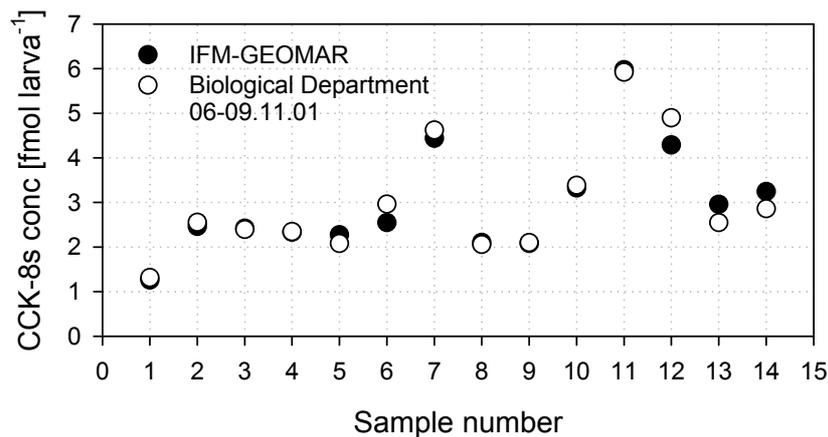
Fig. 14: Comparison of values for CCK concentrations in larval samples, obtained by two different gamma counters available in two different isotope laboratories

2.5.3 Fluorometric analysis of tryptic activity

Each dried pellet of a whole larva or larval body was homogenized in 250-500 µl (factor diluent = 0.5-1) ice-cold TRIS-HCl buffer (0.1 M, pH 8) including $CaCl_2$ x $2H_2O$ (0.02 M), and was centrifuged for 60 min (4110 x g, 4°C) in order to remove tissue fragments. For the buffer Tris(hydroxymethyl)aminomethan ($C_4H_{11}NO_3$, Art. Nr. 8382, M = 121.14, MERCK), 37% HCl, Calciumchlorid-Dihydrat ($CaCl_2$ x $2H_2O$, Art. Nr. 2382, M = 147.02, MERCK) and deionised double-distilled water were used. The supernatants were then used for individual analysis of tryptic activity.

The use of a high specific substrate for trypsin binding to a fluorescent molecule enabled the monitoring of tryptic activity by measuring the increase of the fluorescence per time unit, which is proportional to the amount of active trypsin in the sample. The synthetic substrate used here was Nα-benzoyl-L-arginin-4-methylcoumarinyl-7-amid (BZ-Arg-AMC*HCl, I-1070, MW = 471.94, BACHEM). The substrate solution was prepared fresh prior to each series of measurements. Because of the insolubility of the substrate in water, it was first dissolved in 0.5% Dimethylsulfoxide (DMSO, C_2H_6OS, Art. Nr. 20385, SERVA) of the calculated total volume, and then diluted with TRIS-HCl buffer to a concentration of 0.2 mM.

Tryptic activity is expressed as the amount of hydrolysed substrate (nmol MCA) per minute and larva. It is important to point out that this enzyme activity assay exclusively quantifies the active form of trypsin, i.e. inactive trypsinogen is not quantified (Ueberschär *et al.*, 1992).

The tryptic enzyme activity measurement was carried out in temperature controlled (30°C) two-side-mirror-coated micro-cuvettes (HELLMA), using a computer driven spectral fluorometer (SFM 25, KONTRON) with a four-cuvette holder. The fluorometer was calibrated with distilled water at the beginning of each series of measurements. Before and after use, the micro-cuvettes were handled according to the instructions of the supplier.

For each sample measurement 500 µl of the substrate solution were added to 100 µl of the sample supernatant in one micro-cuvette, and mixed thoroughly. At tryptic activities over the measurable range of the fluorometer, the samples were pre-diluted with TRIS-HCl buffer (factor sample). The emission at 440 nm (excitation 380 nm) was measured in intervals of 2 min over a maximum period of 10 min (Table 10). The fluorescence enhancement, resulting from the enzyme activity was determined by calculating the difference in emission at the beginning and at the end of an interval. The mean fluorescence enhancement was then calculated from 4 interval differences, and converted to nmol MCA per minute and larva via a standard curve, which was produced by using the

fluorophore 7-amino-4-methylcoumarin (MCA, Q-1025, MW = 175.19, BACHEM) in different dilutions with TRIS-HCl buffer (concentrations between 4.6 and 365.3 nM). Fig. 15 shows the mean standard curve and the resulting equation after linear regression fitting that was used to calculate the amount of hydrolysed substrate from the means of fluorescence increase.

Table 10: Constant settings during the measurement of tryptic activity with the computer driven spectral fluorometer (SFM 25, KONTRON)

Number of samples	4
Measurement duration	5 sec.
Number of measurements per sample	4
Time between measurements	2 min.
Excitation	380 nm
Emission	440 nm
Temperature	30 °C
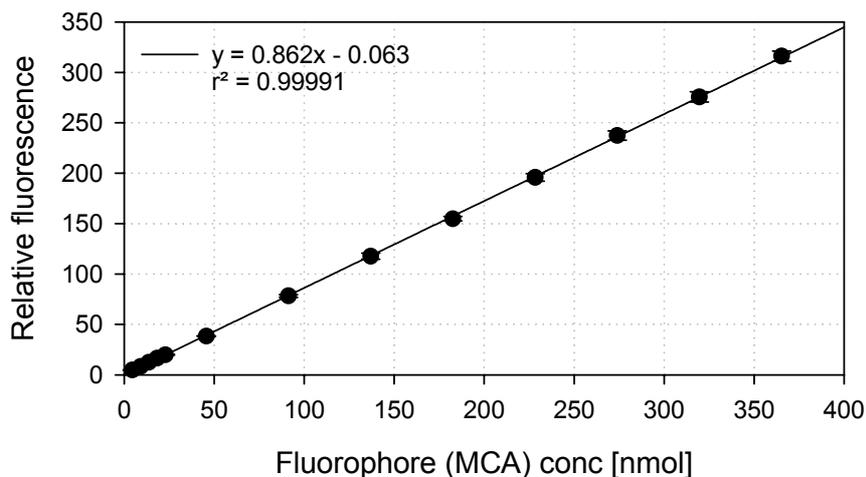
Fig. 15: Standard curve with the fluorophore MCA. The equation resulting from linear regression fitting (p<0.0001) was used to convert the fluorescence increase in tryptic activity, expressed as the amount of hydrolysed substrate (nmol MCA) per minute and larva. Data points represent means of four standard curves ± S.D.

In order to determine the precision of the method, on each day of analysis one sample was measured in triplicates. Through the coefficient of variation from these triplicate measurements a reproducibility of 92.4% was determined.

During the analytical period it is important to ensure the constant functionality of the fluorometer, since e.g. a weak light source (xenon lamp) can affect the measurement

results. Such a control occurred in different time intervals by producing a standard curve with known concentrations of a bovine trypsin (Art. Nr. 37260, 46.5 U/mg, SERVA) mixed at a ratio of 1:10 with bovine albumin (BSA, Art. Nr. 11924, SERVA) in order to prevent autolysis of the trypsin molecules (Fig. 16).
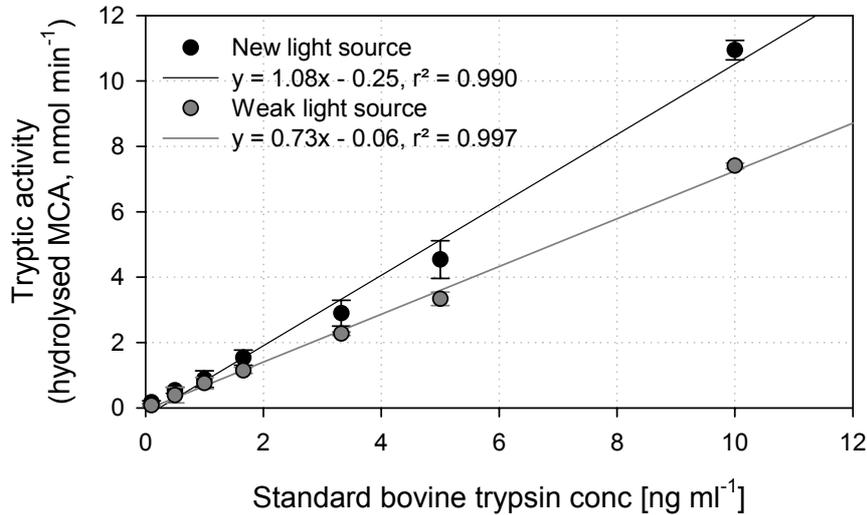
Fig. 16: The black line shows the mean (± S.D.) of three control curves measured within one analytical period, to ensure constant functionality of the fluorometer. The grey line was measured with a weak xenon-lamp, in order to demonstrate the necessity of regular control of the technical equipment. Both curves were fitted by linear function analysis (p<0.0001)

A last control step concerned the combined preparation of samples for the two analytical methods used. In context with the CCK extraction step in methanol, the question arose whether an amount of active trypsin can get lost by dilution in the methanol supernatant. For this purpose some samples were prepared by the regular way as described in § 2.5.1, and tryptic activity was measured in both the pellet and the CCK extract. Fig. 17 shows that only a negligible amount of active trypsin is soluble in methanol.
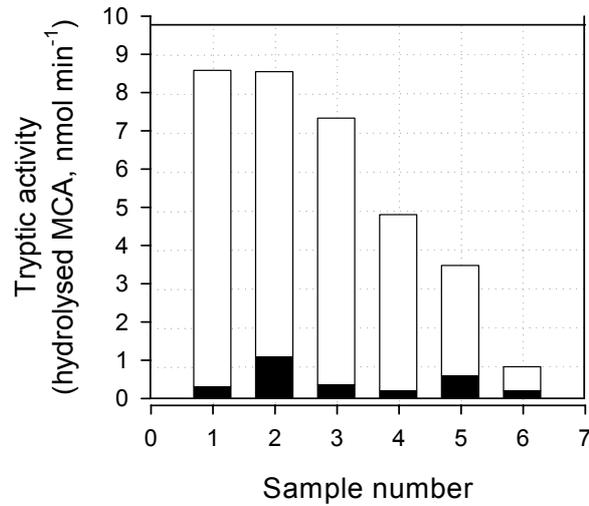
Fig. 17: Tryptic activity in the CCK extract (■) and in the pellet (□) as a control for loss of active trypsin during the CCK extraction step with methanol

2.6 Statistical analysis

Mean values are shown with error bars that represent standard deviations (S.D.). Data were compared by analysis of variance (ANOVA) or the *t*-test after check for normal distribution and equality of variances (Levene's *F*-test). The Tukey *HSD* for unequal n or the Fisher *LSD* post hoc test was used following the ANOVA. The Mann-Whitney *U*-test was used when the criterion of homogenous variances was violated. All statistical analyses were carried out using STATISTICA 6.0 (StatSoft, Tulsa, USA). Differences were considered statistically significant at $p < 0.05$.

3 Results

3.1 Larval mortality, relation to treatment

The halibut larvae used in the present study had a jaw deformity ("gapers"), and were characterised by incapability to feed actively and slower yolk absorption than healthy individuals. Thus, data reported here refer to halibut yolk-sac larvae during the entire experimental period. 26% of the halibut larvae died prior to the start of the experiment, during their transportation from the provider to the experimental facilities. Thereafter, the survival rate in untreated larvae aged 39 to 48 dah, was 38%. It must be noted that while only "gaper" larvae were used in the treatment experiments, the mortality rates refer to both "gaper" and healthy individuals, since all halibut larvae were kept in the same tank and dead specimens were not examined further.

Total mortality due to micro-tube feeding was low with 6%, whereby 15% of dead larvae were counted after micro-injections at 40 dah and none during the next two days of treatment at 44 and 48 dah. In all treatment groups mortalities occurred from 4 h after micro-injection treatment onwards. Treatment related mortalities were lower in the group injected with CCK-8s (2%) than in the control (physiological saline = PS) or PHA group (8% in each).

In herring larvae yolk reserves were observed until 6 dah. Herring larvae that were micro-injected with physiological saline or solutions containing CCK-8s or PHA between 5 and 29 dah showed a total mortality of 25%, whereby the highest daily mortality rate (50%) was recorded at 16 dah. A relationship between mortalities and the incubation period after treatment was not observed. Larvae treated with CCK-8s had the lowest total mortality rate of 16.5%, while that in the PS control group was 27% and in the PHA group 30%.

A total mortality of 7.5% was recorded among untreated larvae that were deprived of food for 4 days. In starving groups of both, untreated and treated herring larvae, the survival was higher in younger than in older larvae. During the starvation experiment with individuals that were treated twice, no great difference in mortality was observed after the second injection (34.5%) when compared with that recorded after the first injection (32%).

Although yolk-like reserves in cod larvae were observed until 13 dah, it is rather difficult to determine the exact date of total yolk-sac absorption by macroscopical observations alone. The mortality rate in untreated cod yolk-sac larvae aged 4-9 dah that were not first fed was 32%. In comparison, the mortality rate in untreated and regularly fed yolk-sac larvae was 47% during the same period, and 71% until day 14 after hatching. The highest mortality rate was recorded at 6 dah.

Among the two experimental groups that were treated by daily immersions in solutions with different CCK-8s concentrations, no great differences in mortality were recorded (0.02 µM CCK-8s: 64.1%, 0.2 µM CCK-8s: 64.8%). However, direct comparisons between mortality rates of untreated and treated cod larvae may not have provided reliable results, since the stocking densities in the rearing and the experimental tanks were not identical.

3.2 Larval growth

3.2.1 Growth data of halibut yolk-sac larvae

Growth trends in standard length and dry weight of halibut yolk-sac larvae are shown in Fig. 18. These "gaper" larvae experienced a visible stimulation through the administered food, but did not feed actively due to the jaw deformity. All experimental groups showed similar growth data, since the treatment applied was only a short-term one, meaning that each larva was micro-injected and sampled on the same day. Slight differences on certain days can only be attributed to the individual larval variability.

Standard length increment more or less stagnated between 40 and 44 dah but showed an obvious decline on day 48 after hatching. In contrast, dry weight decreased throughout the experimental period with daily rates of -0.005 (untreated larvae), -0.02 (PS and CCK-8s group) and -0.01 (PHA group) mg/d.
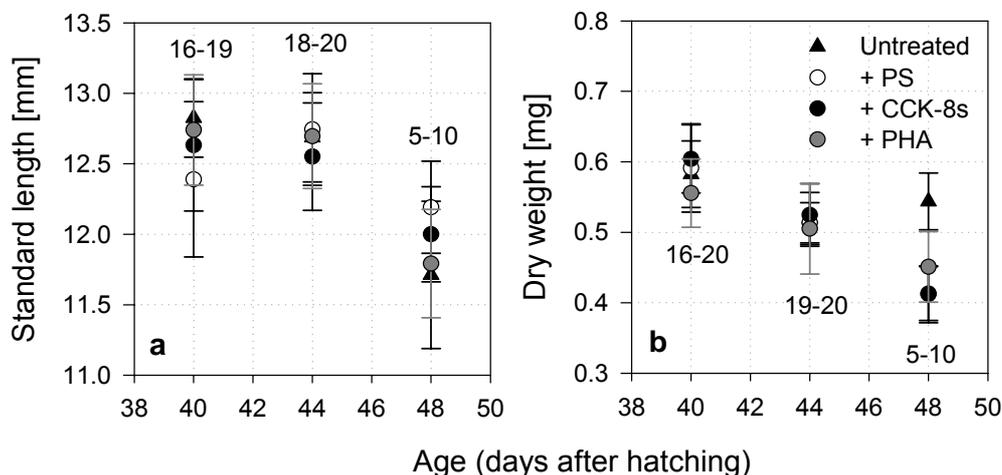
Fig. 18: Development of length (a) and weight (b) of "gaper" halibut yolk-sac larvae during the experimental period. Data are given as means (symbols) with standard deviations (bars) and represent four experimental groups: untreated controls, treated controls micro-injected with physiological saline (PS), and larvae treated with the hormone cholecystokinin (CCK-8s) or the plant protein phytohemagglutinin (PHA). n = number of individual measurements per mean

3.2.2   Growth data of herring larvae

In Fig. 19 data of dry weight in relation to standard length of herring larvae are shown for each experimental group. Curves were fitted by power function analysis and were highly significant (p<0.0001) in all groups. Because of the large number of samples and the time consuming processing for the chemical analysis, the dry weight – in contrast to the standard length - was initially not determined for every individual herring larva. Still, in order to later allow a weight specific presentation of results, the equations from the fitted curves were used to back calculate missing larval weights.

In Fig. 20 standard length, as well as empirical and calculated dry weight data were plotted versus the age of herring larvae from each of the four experimental groups. In all groups standard length and dry weight showed a significantly linear and exponential growth with age, respectively. Slight differences, especially from day 26 after hatching onwards, can be attributed to the individual variability, which increased with age. In total all groups showed a comparable length and weight growth with 10.13 ± 0.40 mm and 0.18 ± 0.08 mg (n = 71) at the beginning (5 dah) and 19.05 ± 1.43 mm and 1.28 ± 0.40 mg (n = 86) at the end (29 dah) of the experiment. For the period of 5-29 dah the daily growth rate in standard length was 0.33 mm/d in the PS group and 0.38 mm/d in all the other groups. The daily growth rate in dry weight was between 0.04 and 0.05 mg/d in all four groups.

Fig. 21 shows length data of starving larvae from each experimental group in comparison to the length of the respective fed individuals during the same developmental stage. Similarly, Fig. 22 compares the dry weight between starving and fed larvae of each experimental group with age. In the starvation experiments with all four groups – although short – a stagnation or even decrease was observed in the length and weight data 24-48 hours after food deprivation. The difference between regularly fed and starving larvae was more obvious towards the end of the experiment in later developmental stages. In the case e.g. of untreated larvae during the first starvation approach from 8 to 11 dah no statistically significant differences were found in length and weight between fed and starving larvae; from16 to 19 dah only standard length was significantly different; and from 25 to 28 dah both standard length and dry weight were significantly different between fed and starved individuals.
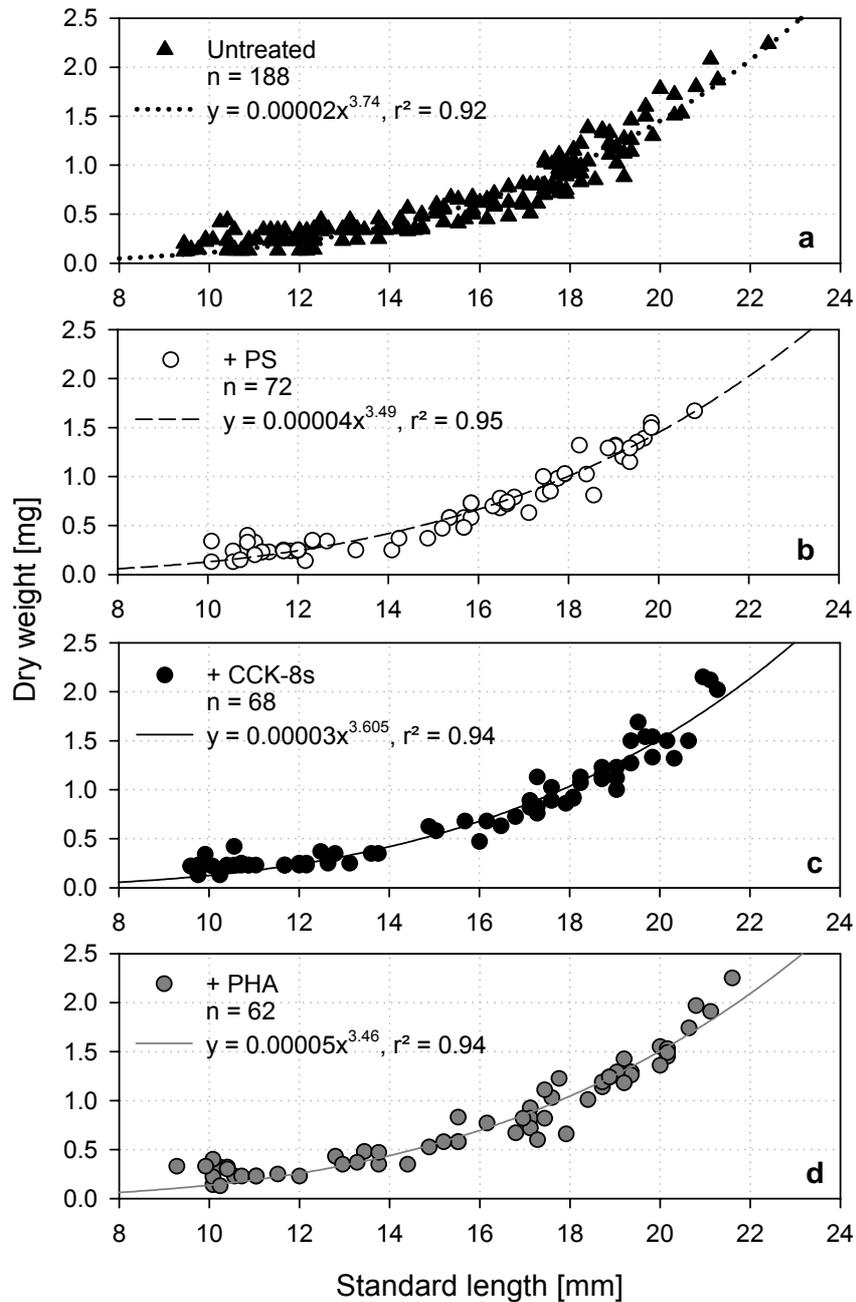
Fig. 19: Relation between standard length and dry weight of herring larvae. Data show individual measurements and represent four experimental groups: untreated controls (a), treated controls micro-injected with physiological saline (PS) (b), and larvae treated with the hormone cholecystokinin (CCK-8s) (c) or the plant protein phytohemagglutinin (PHA) (d). Untreated larvae were sampled daily from 5 to 29 dah, while samples of micro-injected larvae were taken only on treatment days (at 5, 6, 11, 16, 20, 25 and 29 dah). The curves were fitted by power function analysis (p<0.0001 in all groups). n = number of individual measurements
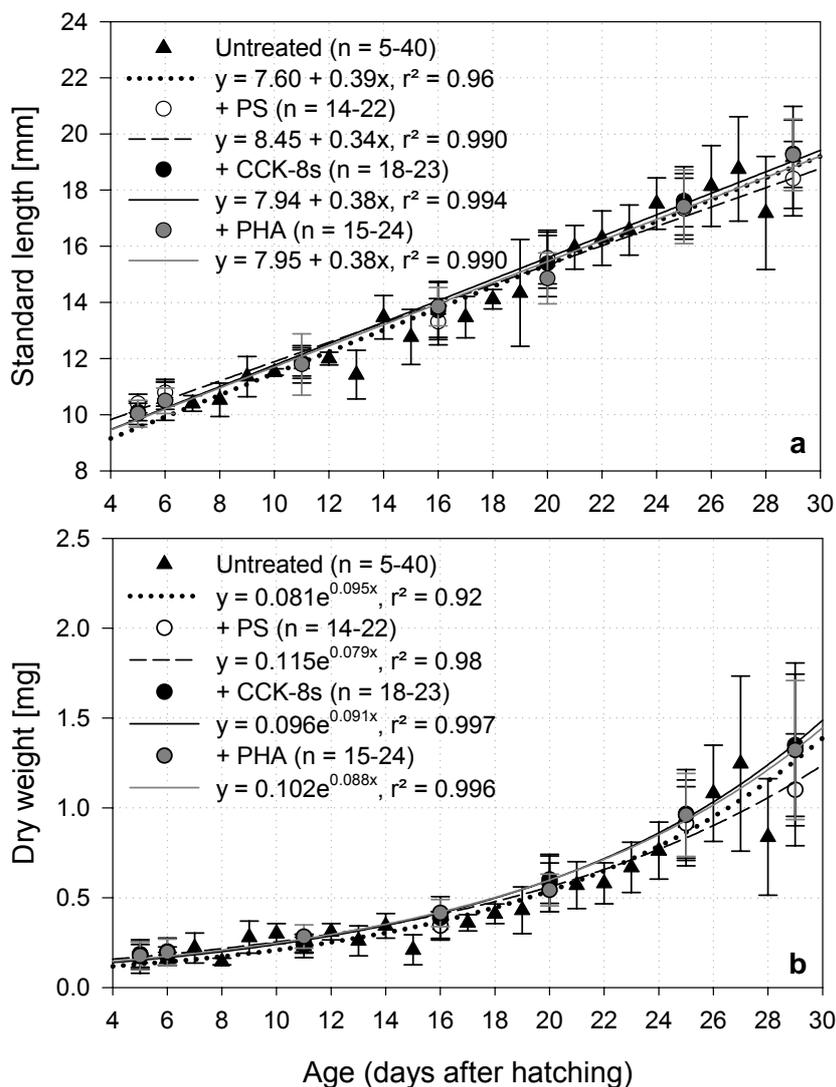
Fig. 20: Development of length (a) and weight (b) of herring larvae during the experimental period. Data are given as means (symbols) with standard deviations (bars) and represent four experimental groups: untreated controls, treated controls micro-injected with physiological saline (PS), and larvae treated with the hormone cholecystokinin (CCK-8s) or the plant protein phytohemagglutinin (PHA). The curves were fitted to the length data by linear regression (p<0.0001 in all groups) and to the weight data by exponential function analysis (p<0.0001 in all groups). n = number of individual measurements per mean
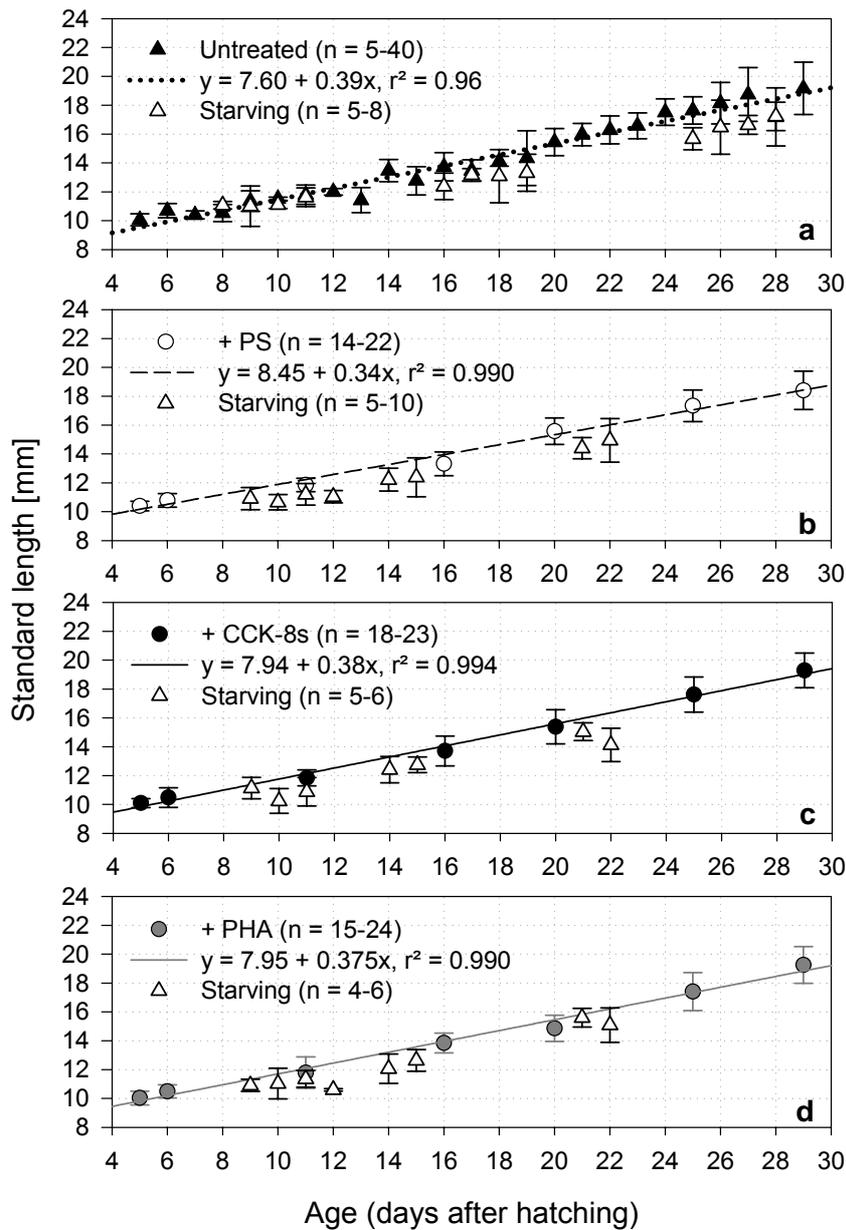
Fig. 21: Comparison of length data between fed and starving herring larvae during the experimental period. Data are given as means (symbols) with standard deviations (bars) and represent four experimental groups with their starving subgroups: untreated controls (a), treated controls micro-injected with physiological saline (PS) (b), and larvae treated with the hormone cholecystokinin (CCK-8s) (c) or the plant protein phytohemagglutinin (PHA) (d). Individuals in each starving experiment were deprived of food one day prior to the first sampling and analysis. Starving larvae of the treated groups (b-d) were injected with the respective solution once, deprived of food and then sampled over the following days. The curves were fitted by linear regression (p<0.0001 in all groups). n = number of individual measurements per mean
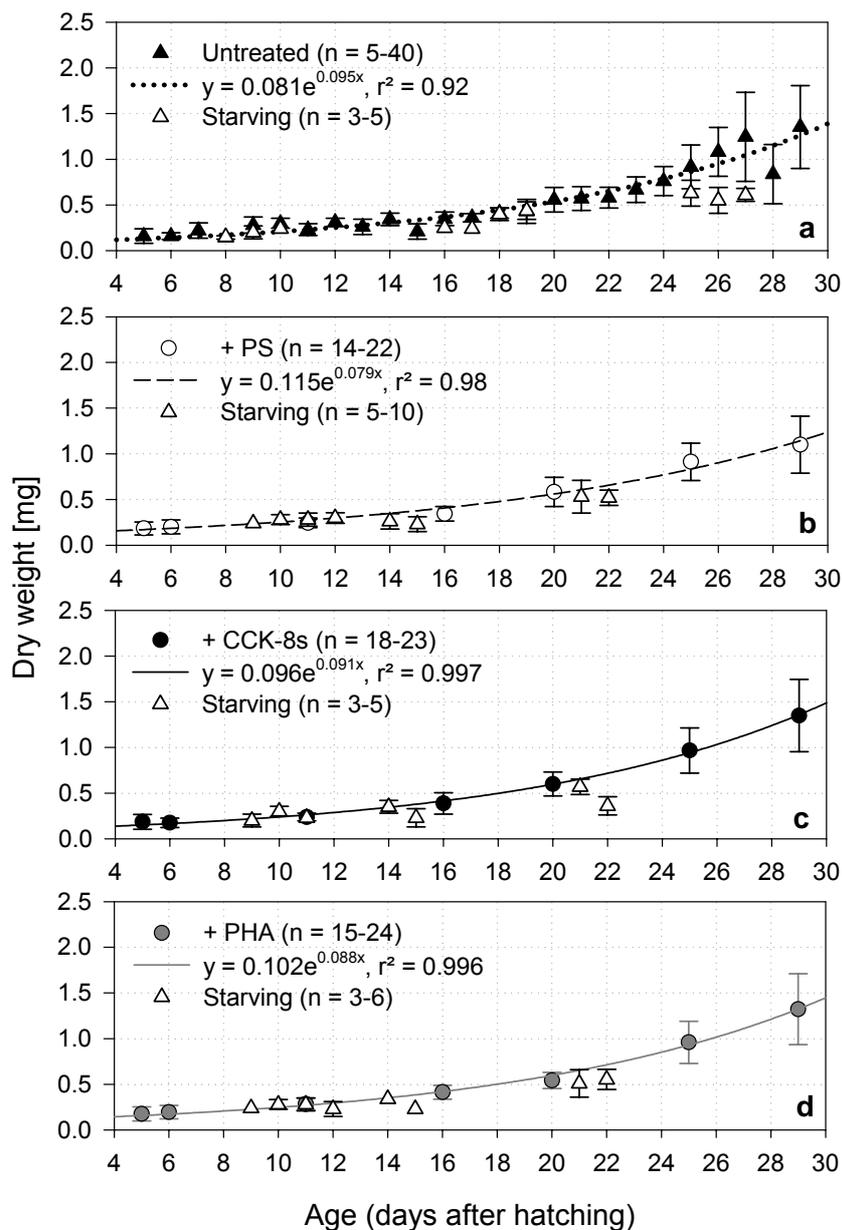
Fig. 22: Comparison of weight data between fed and starving herring larvae during the experimental period. Data are given as means (symbols) with standard deviations (bars) and represent four experimental groups with their starving subgroups: untreated controls (a), treated controls micro-injected with physiological saline (PS) (b), and larvae treated with the hormone cholecystokinin (CCK-8s) (c) or the plant protein phytohemagglutinin (PHA) (d). Individuals in each starving experiment were deprived of food one day prior to the first sampling and analysis. Starving larvae of the treated groups (b-d) were injected with the respective solution once, deprived of food and then sampled over the following days. The curves were fitted by exponential function analysis (p<0.0001 in all groups). n = number of individual measurements per mean

3.2.3 Growth data of cod larvae

Cod larvae aged 2 dah had a mean standard length of 4.00 ± 0.18 mm and a mean dry weight of 0.27 ± 0.03 mg (n = 6).

The standard length of cod larvae from all experimental groups is shown versus the age in Fig. 23. In fed untreated and long-term treated cod larvae standard length showed an increment of 0.1-0.2 mm/d until day 6 after hatching and stagnation thereafter. Significant differences in length increments were observed between untreated fed larvae and untreated larvae that were not first fed, relying only on their yolk reserve (Fig. 23a).
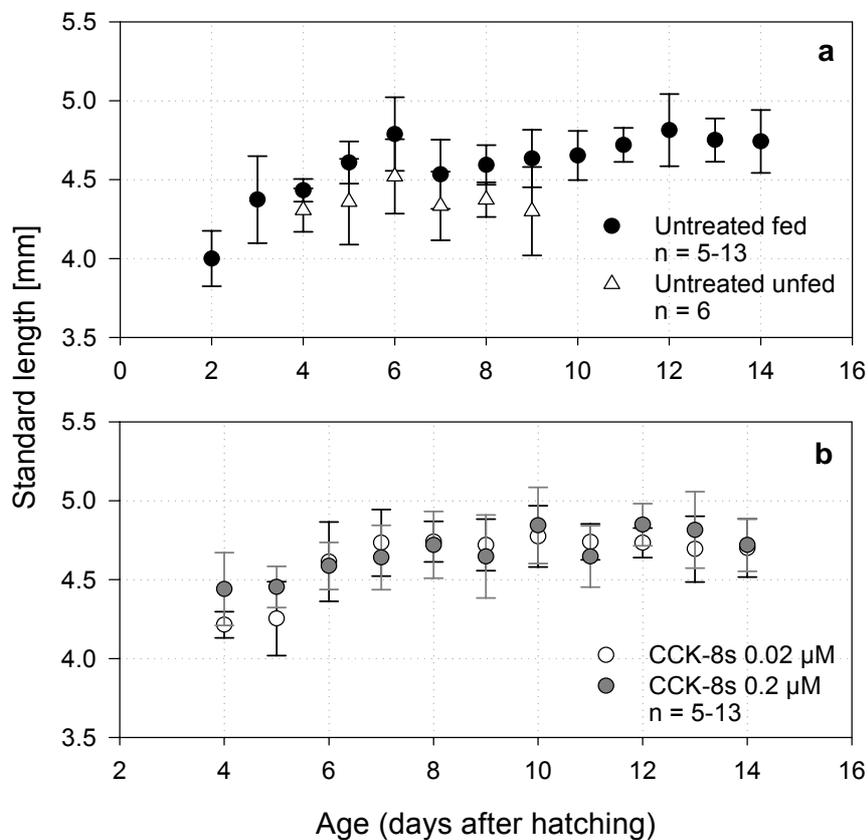
Fig. 23: Standard length in relation to the age of cod larvae: a) comparison between untreated fed controls and untreated larvae that were not first fed, b) larvae treated with the hormone cholecystokinin (CCK-8s) by daily immersions in a 0.02 µM or 0.2 µM solution. Data are given as means (symbols) with standard deviations (bars). n = number of individual measurements per mean

Fig. 24 shows the dry weight of cod larvae from all experimental groups in relation to the age. In contrast to the larval length which represented a quite narrow range but was significantly higher in fed than in unfed larvae, the dry weight data revealed no significant

difference between these two groups (Fig. 24a). The development of dry weight showed two significant decreases in all experimental groups: the first pronounced weight decrease took place at 5 dah in treated and one day later in untreated larvae, while the second one occurred simultaneously in all groups at 10 dah; both were followed by days with slight weight increments. A switch in the feeding schedule and the size or species of feeding organisms were not the reason for these weight losses, since the only change occurred on day 13 after hatching when wild zooplankton was offered additionally to the enriched rotifers.

With exception of the significant weight difference between untreated and treated larvae mentioned on day 5 after hatching, the hormonal treatment did not seem to have a more dominant effect on growth than the food.
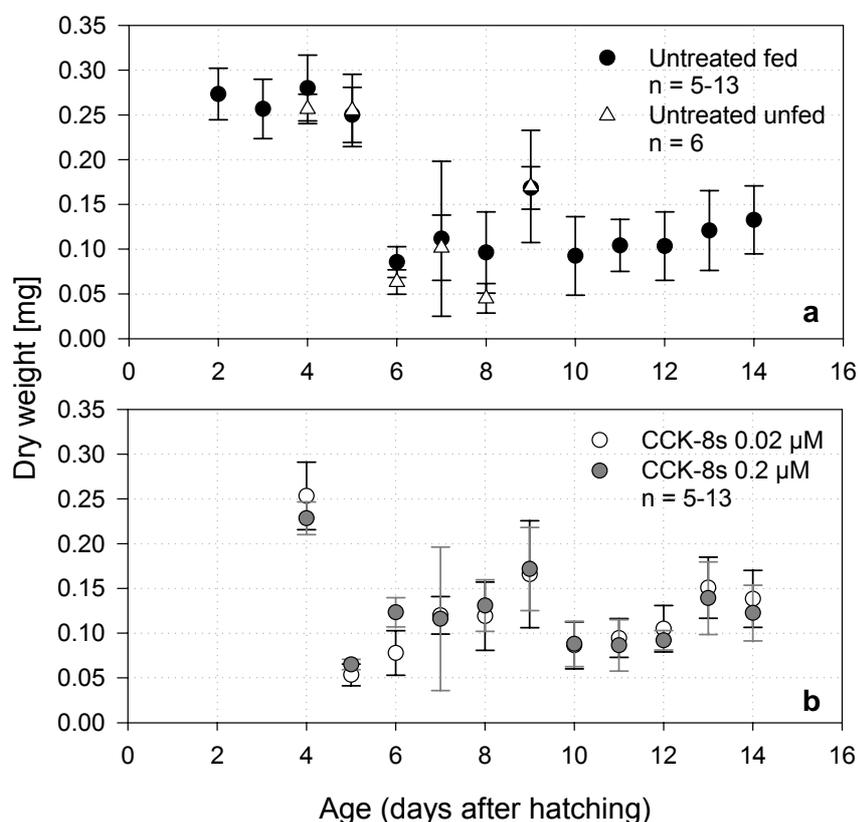
Fig. 24: Dry weight in relation to the age of cod larvae: a) comparison between untreated fed controls and untreated larvae that were not first fed, b) larvae treated with the hormone cholecystokinin (CCK-8s) by daily immersions in a 0.02 µM or 0.2 µM solution. Data are given as means (symbols) with standard deviations (bars). n = number of individual measurements per mean
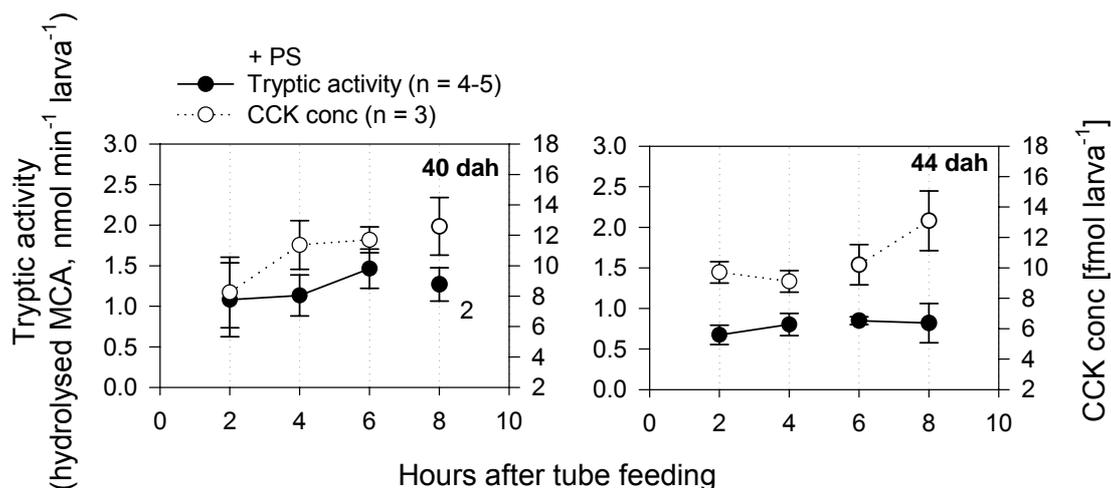
3.3   CCK content and tryptic activity in halibut yolk-sac larvae

As previously described (§ 3.2.1), standard length of halibut larvae showed a decrease on day 48 after hatching, suggesting an insufficient nutritional supply by the yolk reserve. At this point, a predominant effect of the "gaper" disease and consequent incapability of active feeding on the larval physiology could not be excluded. Thus, only halibut larvae aged 40 and 44 dah were included in the chemical analysis.

In Fig. 25 larval CCK content and tryptic activity of untreated halibut are shown in relation to different sampling times on day 40 and 44 after hatching. Both parameters showed an opposite trend over the day. The same trend was observed in the CCK level and tryptic activity of larvae micro-injected with physiological saline (Fig. 26) and those treated with PHA (Fig. 28), when plotted versus the incubation period after treatment. In contrast, this opposite pattern was not as clear in individuals of the CCK-8s group (Fig. 27). An identical trend in the development of CCK concentration or tryptic activity over the day or over the incubation period after treatment, between the two sampling days was not evident. However, when considering larvae aged 40 dah the CCK content increased in all groups until at least 4 hours after the first sampling, while tryptic activity continuously decreased in untreated controls, but increased in the treated groups between 4 and 6 hours after tube feeding. In all experimental groups the values for CCK content and tryptic activity were lower on day 44 than on day 40 after hatching.
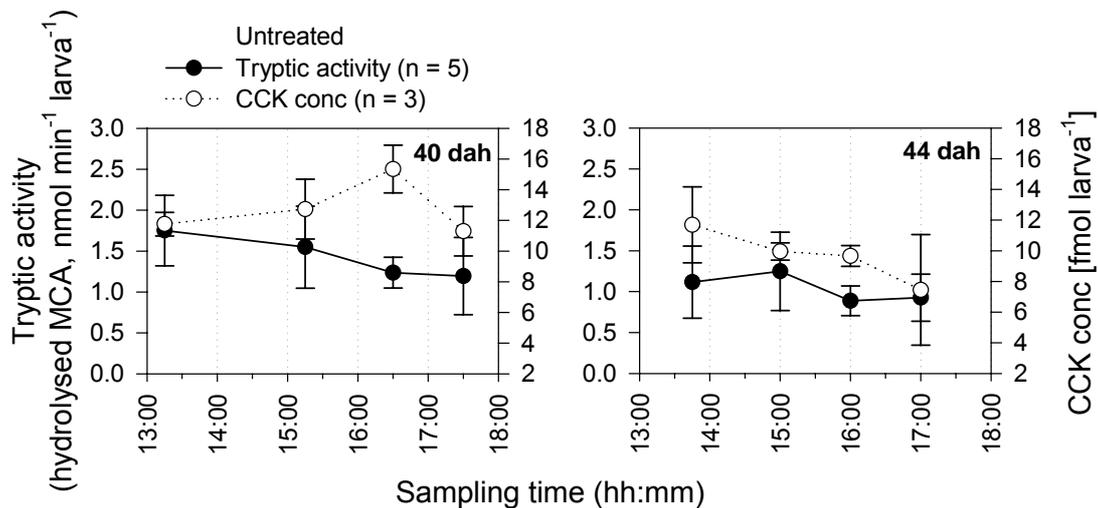
Fig. 25: Untreated "gaper" halibut yolk-sac larvae: tryptic activity and CCK content in relation to the sampling time at 40 and 44 dah. Data are given as means (symbols) with standard deviations (bars). n = number of individual measurements per mean

Fig. 26: "Gaper" halibut yolk-sac larvae micro-injected with physiological saline (PS): tryptic activity and CCK content in relation to the incubation period after treatment at 40 and 44 dah. Data are given as means (symbols) with standard deviations (bars). Means that are not connected with a line represent not successive sampling times (e.g. a larva 8 h after treatment can have been micro-injected and sampled earlier than one 6 h after treatment). n = number of individual measurements per mean
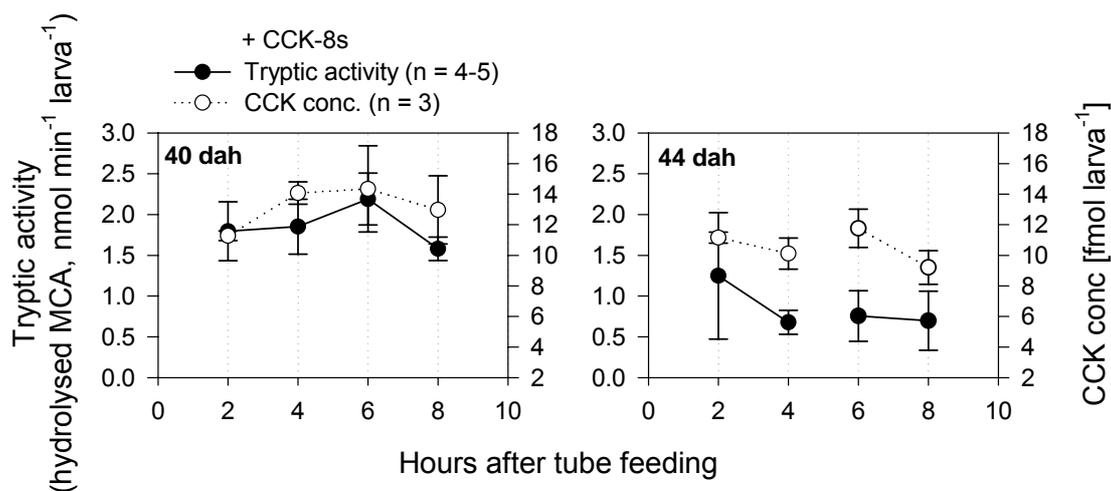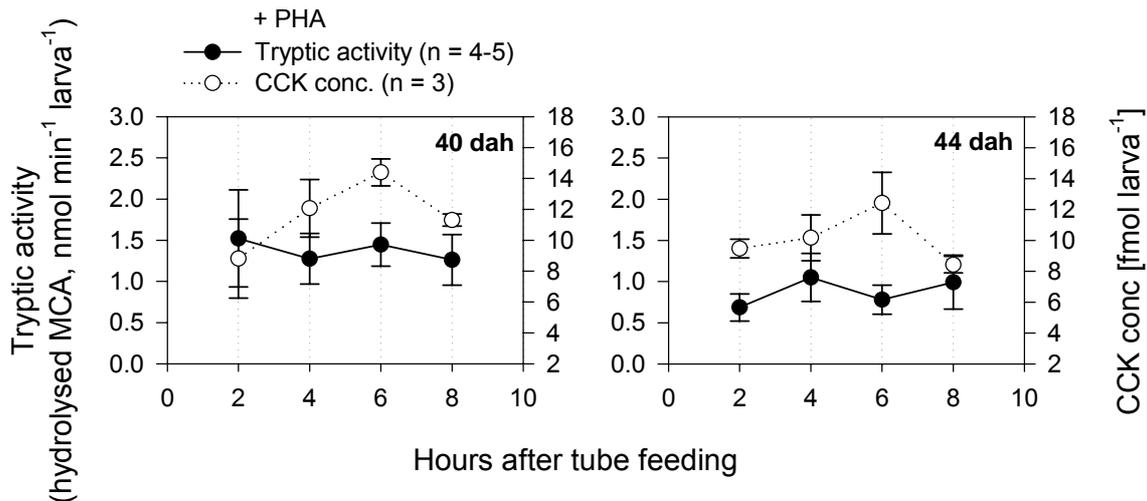

Fig. 27: "Gaper" halibut yolk-sac larvae treated with the hormone cholecystokinin (CCK-8s): tryptic activity and CCK content in relation to the incubation period after application of a micro-injection at 40 and 44 dah. Data are given as means (symbols) with standard deviations (bars). Means that are not connected with a line represent not successive sampling times (e.g. a larva 8 h after treatment can have been micro-injected and sampled earlier than one 6 h after treatment). n = number of individual measurements per mean

Fig. 28: "Gaper" halibut yolk-sac larvae treated with the plant protein phytohemagglutinin (PHA): tryptic activity and CCK content in relation to the incubation period after application of a micro-injection at 40 and 44 dah. Data are given as means (symbols) with standard deviations (bars). n = number of individual measurements per mean

In order to decide whether the micro-injection treatment itself had an effect on the larvae, or if the untreated larval group could be used just as well as a control for treated larvae as the group micro-injected with physiological saline, a comparison of CCK levels and tryptic activities between the two controls was applied. In Fig. 29 the CCK content between untreated and treated controls is compared during different sampling times at 40 and 44 dah, while Fig. 30 shows the same comparisons for data of tryptic activity. In both parameters significant differences were found between untreated larvae and those micro-injected with physiological saline. The values of CCK content and of tryptic activity were lower in the PS group than in untreated larvae, until at least 4 hours after treatment. Thus, conclusions about the effect of treatment with CCK-8s or PHA should be based only on comparisons with the PS group as a control.
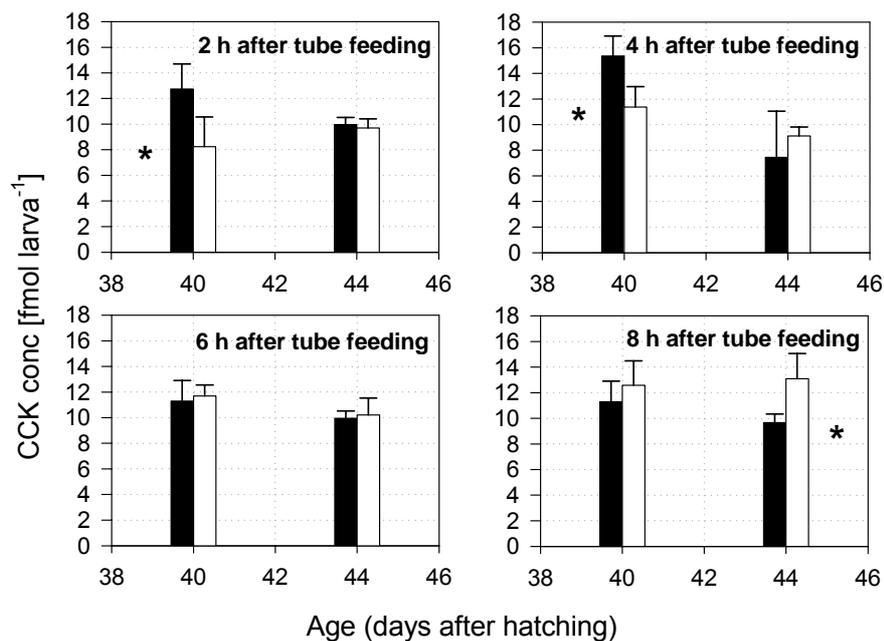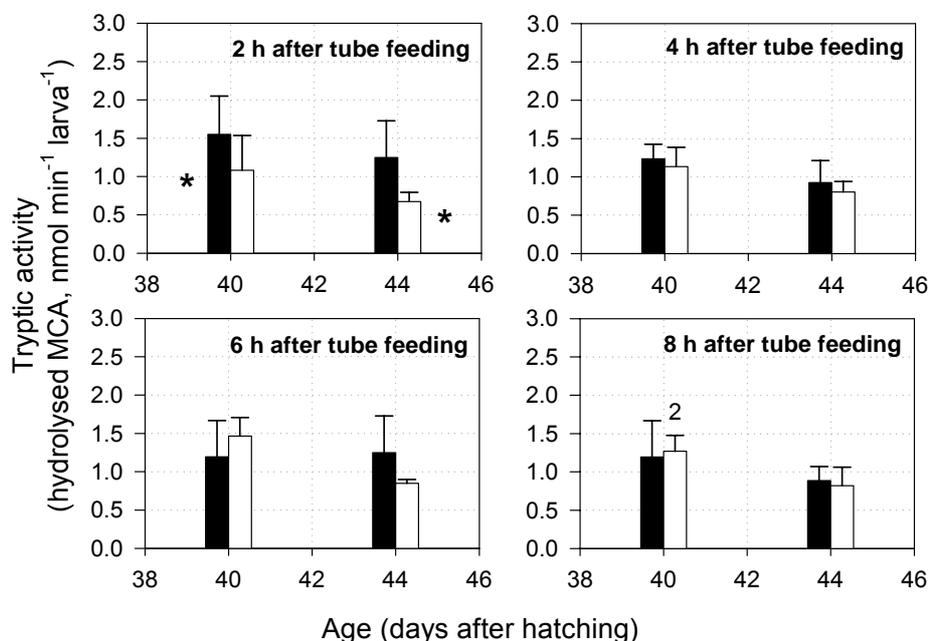
Fig. 29: Comparison of CCK content between "gaper" halibut yolk-sac larvae that were not treated (■) and those micro-injected with physiological saline (PS) (□), at 40 and 44 dah. Data are given as means (symbols) with standard deviations (bars). The time after treatment marking each graph refers to the incubation period of the PS-group, while untreated larvae were sampled at the respective time of the day. Pairs with significant differences are marked by an asterisk. n = 3 individual measurements per mean

Fig. 30: Comparison of tryptic activity between "gaper" halibut yolk-sac larvae that were not treated (■) and those micro-injected with physiological saline (PS) (□), at 40 and 44 dah. Data are given as means (symbols) with standard deviations (bars). The time after treatment marking each graph refers to the incubation period of the PS-group, while untreated larvae were sampled at the respective time of the day. Pairs with significant differences are marked by an asterisk. n = 4-5 individual measurements per mean

As mentioned above, CCK as well as tryptic activity levels declined towards 44 dah. Considering also the decreasing weight data, "gaper" halibut larvae older than 40 dah did not show a good nutritional condition. Since the aim of this study was to evaluate the effects of CCK-8s and PHA on larvae with a sufficient nutritional supply and not on starving individuals, for the direct comparison of treated groups only data of larvae aged 40 dah were used. At that stage, the effects of the test solutions aught to reflect results from normal first fed larvae.

Fig. 31 and 32 compare the CCK content and the tryptic activity, respectively between larvae treated with CCK-8s or PHA and individuals from the PS control group at the age of 40 dah. Both, the CCK content and the tryptic activity in larvae treated with CCK-8s increased until 6 hours after treatment, reaching 14.34 ± 2.81 fmol (n = 3) and 2.19 ± 0.32 nmol/min (n = 4), respectively and were higher than in the PS-controls (Fig. 31a and 32a). At 8 hours after treatment both parameters fell to levels near the ones measured in the PS group. The PHA treatment caused a significantly higher CCK level than that in the PS controls not before 6 hours after treatment, which fell again two hours later (Fig. 31b). In tryptic activity no differences were observed between PHA and the PS control group until 8 hours after the micro-injections (Fig. 32b).

Fig. 31: Comparison of CCK content between "gaper" halibut yolk-sac larvae that were micro-injected only with physiological saline (PS) and those treated with the hormone cholecystokinin (CCK-8s) (a) or the plant protein phytohemagglutinin (PHA) (b), at 40 dah. Data are related to the incubation period after treatment and are given as means (symbols) with standard deviations (bars). Means that are not connected with a line represent not successive sampling times (e.g. a larva 8 h after treatment can have been micro-injected and sampled earlier than one 6 h after treatment). Pairs with significant differences are marked by an asterisk. n = 3 individual measurements per mean
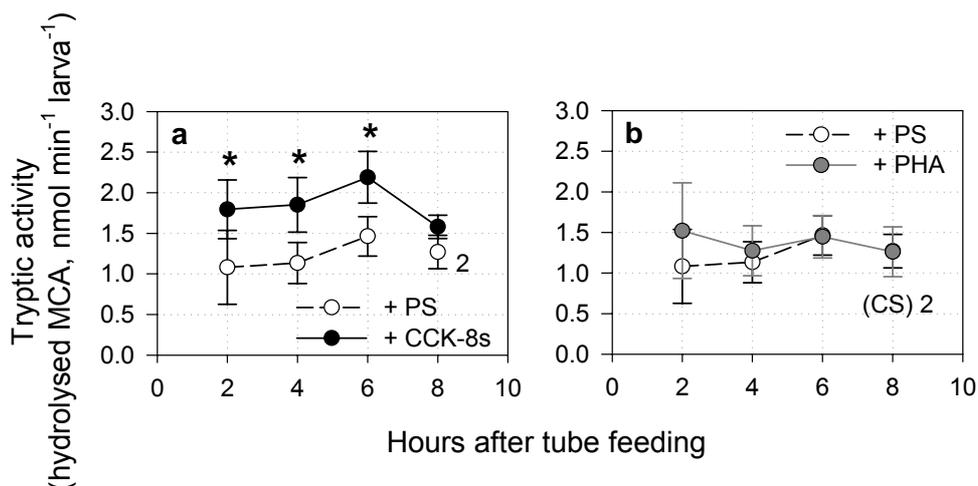
Fig. 32: Comparison of tryptic activity between "gaper" halibut yolk-sac larvae that were micro-injected only with physiological saline (PS) and those treated with the hormone cholecystokinin (CCK-8s) (a) or the plant protein phytohemagglutinin (PHA) (b), at 40 dah. Data are related to the incubation period after treatment and are given as means (symbols) with standard deviations (bars). Means that are not connected with a line represent not successive sampling times (e.g. a larva 8 h after treatment can have been micro-injected and sampled earlier than one 6 h after treatment). Pairs with significant differences are marked by an asterisk. n = 4-5 individual measurements per mean

3.4   CCK content and tryptic activity in herring larvae

Data of CCK content and tryptic activity plotted versus the age of untreated herring individuals that were sampled daily (Fig. 33a), showed opposite peaks from the time yolk-sac absorption was completed (6-7 dah) to the end of the experiment at 29 dah: high CCK levels corresponded to low tryptic activities and vice versa. When considered in total though, both parameters showed an overall increasing tendency in their values with age. The daily interaction pattern was also evident when CCK content and tryptic activity were related to the individual larval dry weight (Fig. 33b). While dry weight specific CCK concentrations slightly decreased with age, dry weight specific tryptic activities seemed to oscillate around a steady level range.
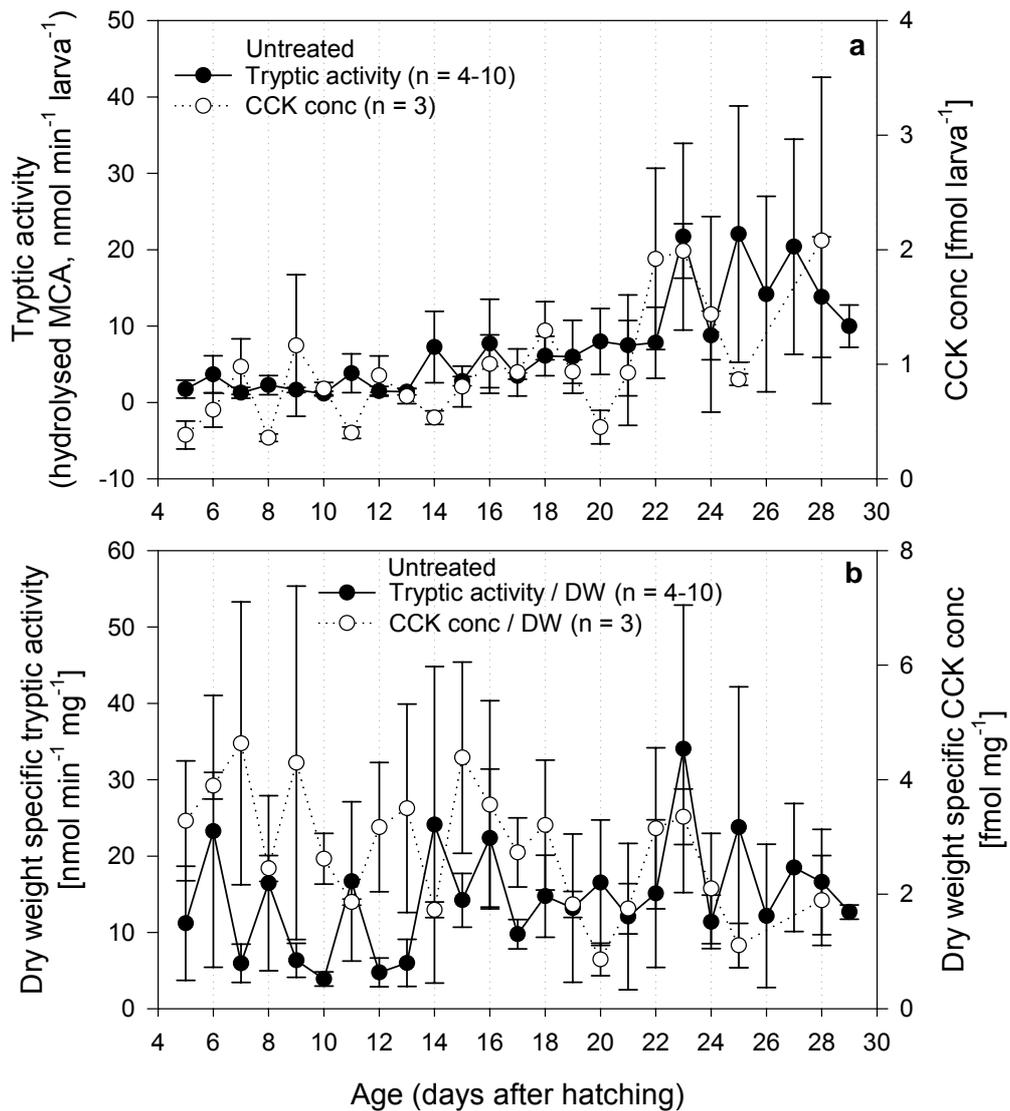
Fig. 33: Development of tryptic activity and CCK content (a) as well as of weight specific tryptic activity and weight specific CCK content (b) of untreated herring larvae during the experimental period. Data are given as means (symbols) with standard deviations (bars) and represent larvae that were sampled once daily between 12:00 and 13:30 h, before the feeding time of the day. n = number of individual measurements per mean

The information about the feedback relationship between CCK and tryptic activity shown due to daily sampling, was not detectable when samples were not taken every day and data presented wider time intervals (Fig. 34): at selected days during the experimental period the development of CCK content and tryptic activity in untreated herring larvae showed the same trends with age, irrespective of the sampling time during the day.
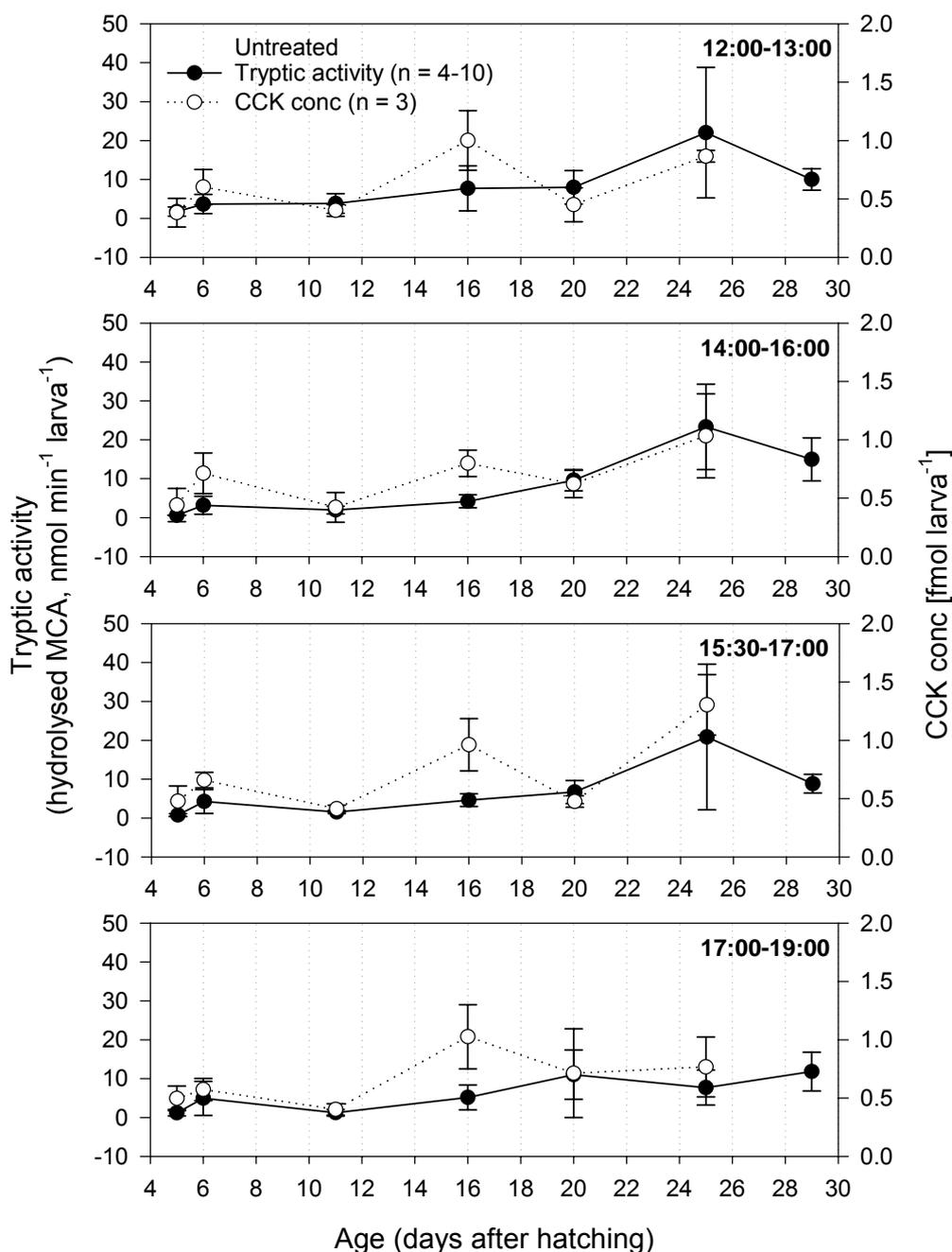
Fig. 34: Untreated herring larvae: tryptic activity and CCK content at selected days during the experimental period. Each graph represents one sampling time. Data are given as means (symbols) with standard deviations (bars). n = number of individual measurements per mean

Larvae treated with CCK-8s or PHA at 5, 6, 11, 16, 20, 25 and 29 dah showed the same development in their CCK content and tryptic activity as the untreated individuals (Fig. 35 and 36). Similarly to the untreated group, the trends of CCK content and tryptic activity in treated larvae were the same throughout the different incubation times after treatment.
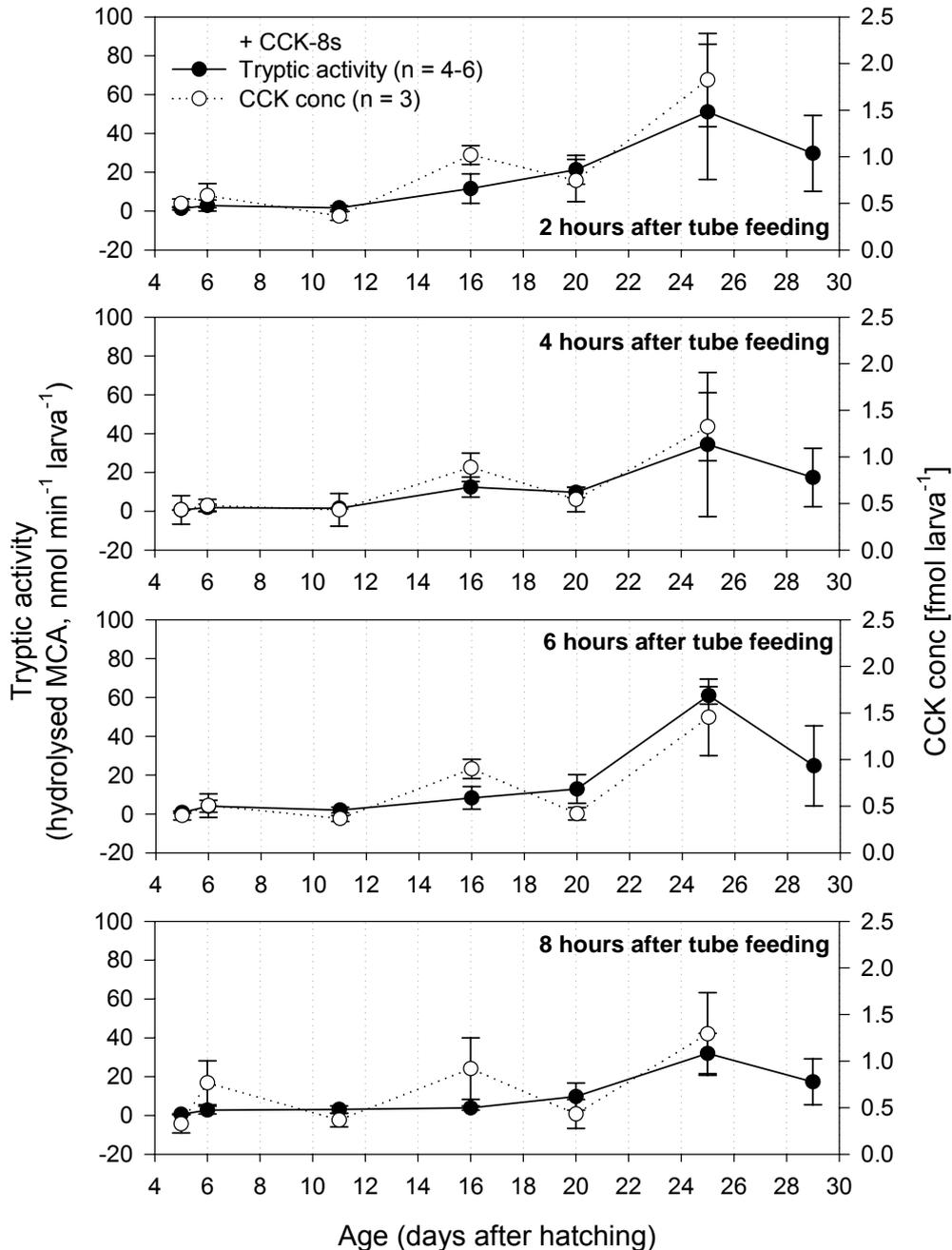
Fig. 35: Herring larvae treated with the hormone cholecystokinin (CCK-8s): tryptic activity and CCK content at selected days during the experimental period. Each graph represents one incubation period after application of a micro-injection. Data are given as means (symbols) with standard deviations (bars). n = number of individual measurements per mean
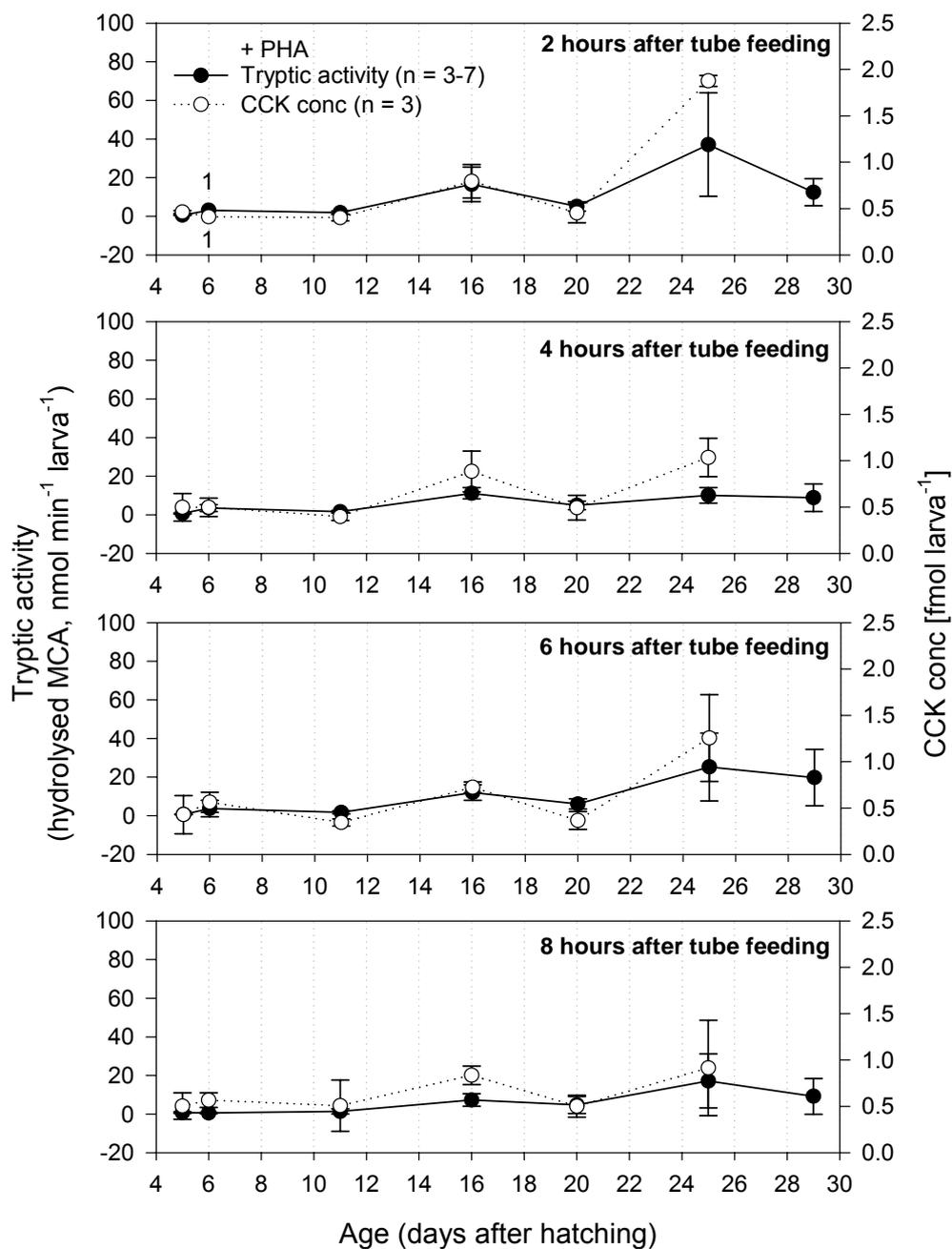
Fig. 36: Herring larvae treated with the plant protein phytohemagglutinin (PHA): tryptic activity and CCK content at selected days during the experimental period. Each graph represents one incubation period after application of a micro-injection. Data are given as means (symbols) with standard deviations (bars). n = number of individual measurements per mean

In all approaches with starving larvae, either from the untreated batch or from the treated groups, stagnating or even declining CCK concentrations and tryptic activities were observed 24-48 hours after food deprivation, showing that both parameters react as short-term indicators to changes in the food supply (Fig. 37 and 38).
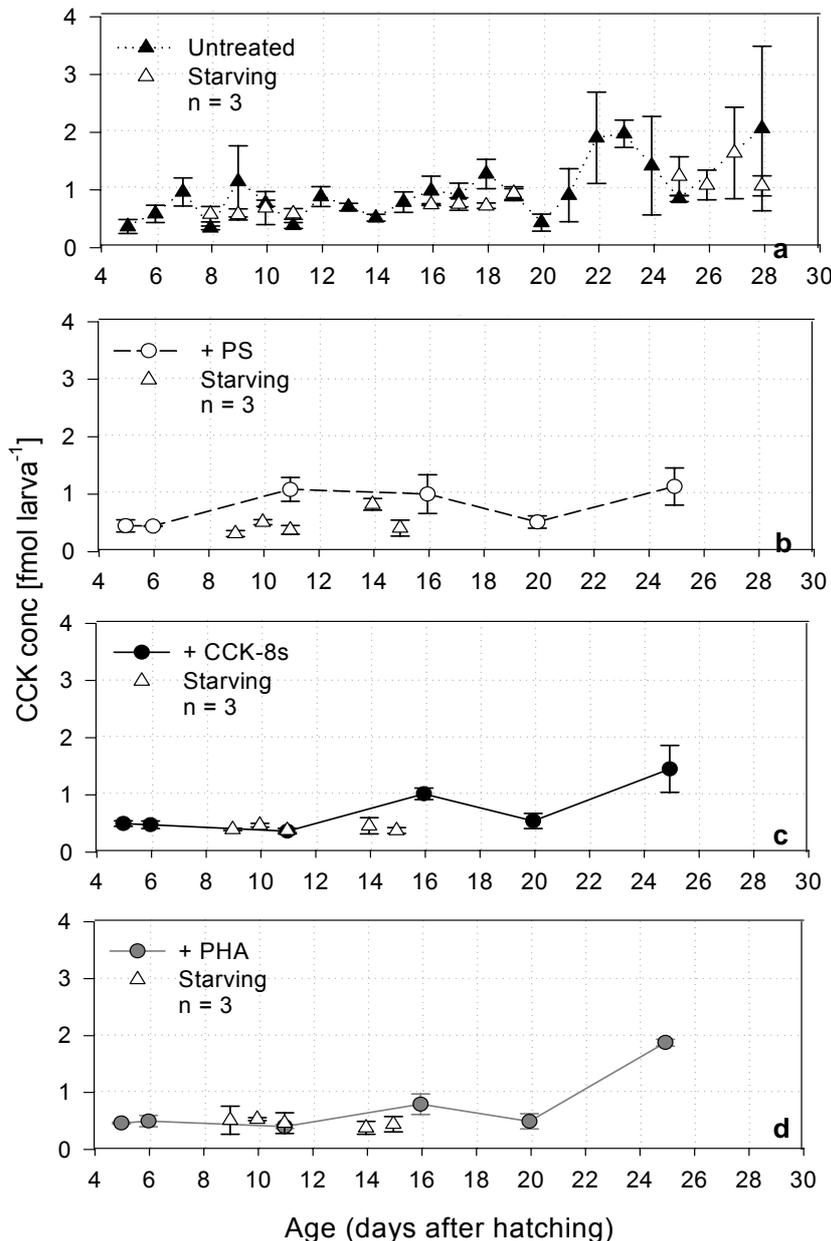
Fig. 37: Comparison of CCK content between fed and starving herring larvae during the experimental period. Data are given as means (symbols) with standard deviations (bars) and represent four experimental groups with their starving subgroups: untreated controls (a), treated controls micro-injected with physiological saline (PS) (b), and larvae treated with the hormone cholecystokinin (CCK-8s) (c) or the plant protein phytohemagglutinin (PHA) (d). Individuals in each starving experiment were deprived of food one day prior to the first sampling and analysis. Starving larvae of the treated groups (b-d) were injected with the respective solution once, deprived of food and then sampled over the following days. n = number of individual measurements per mean
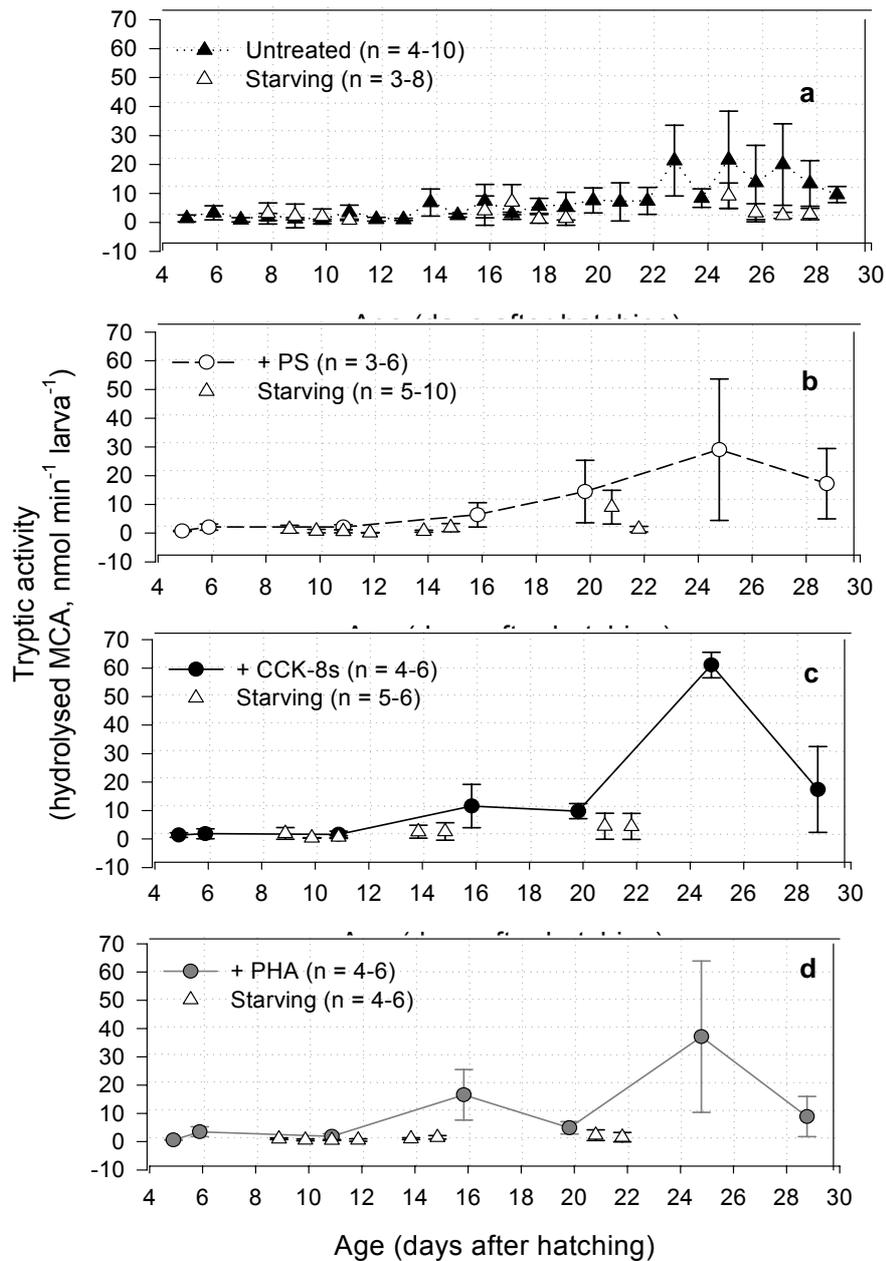
Fig. 38: Comparison of tryptic activity between fed and starving herring larvae during the experimental period. Data are given as means (symbols) with standard deviations (bars) and represent four experimental groups with their starving subgroups: untreated controls (a), treated controls micro-injected with physiological saline (PS) (b), and larvae treated with the hormone cholecystokinin (CCK-8s) (c) or the plant protein phytohemagglutinin (PHA) (d). Individuals in each starving experiment were deprived of food one day prior to the first sampling and analysis. Starving larvae of the treated groups (b-d) were injected with the respective solution once, deprived of food and then sampled over the following days. n = number of individual measurements per mean

In Fig. 39 CCK content and tryptic activity of untreated herring larvae are shown in relation to the sampling time at selected days, during which multiple sampling was performed. The sampling times on each day correspond to the times larvae of the treated groups were sampled after application of a micro-injection and incubation. For treated groups the data of CCK content and tryptic activity on each treatment day are shown in relation to the incubation period after micro-injection of physiological saline as a treatment control (Fig. 40), or after treatment with CCK-8s (Fig. 41) or PHA (Fig. 42).

In all groups the values for CCK content and tryptic activity varied greatly among different developmental stages, and this is evident by the different scaling of the y-axis on each daily graph. A homogenous pattern in the daily trends of CCK content and tryptic activity among different days was not evident, neither in untreated individuals, nor in those micro-injected with physiological saline, CCK-8s or PHA solution. The development of CCK content and tryptic activity during the day or during the time after treatment for untreated and treated larvae, respectively was not even the same on 5 and 6 dah; at these stages yolk reserve was still present and a more regular metabolic reaction could be assumed in contrast to older larvae that depend on exogenous food and show a greater individual variability in the time of actual food intake.
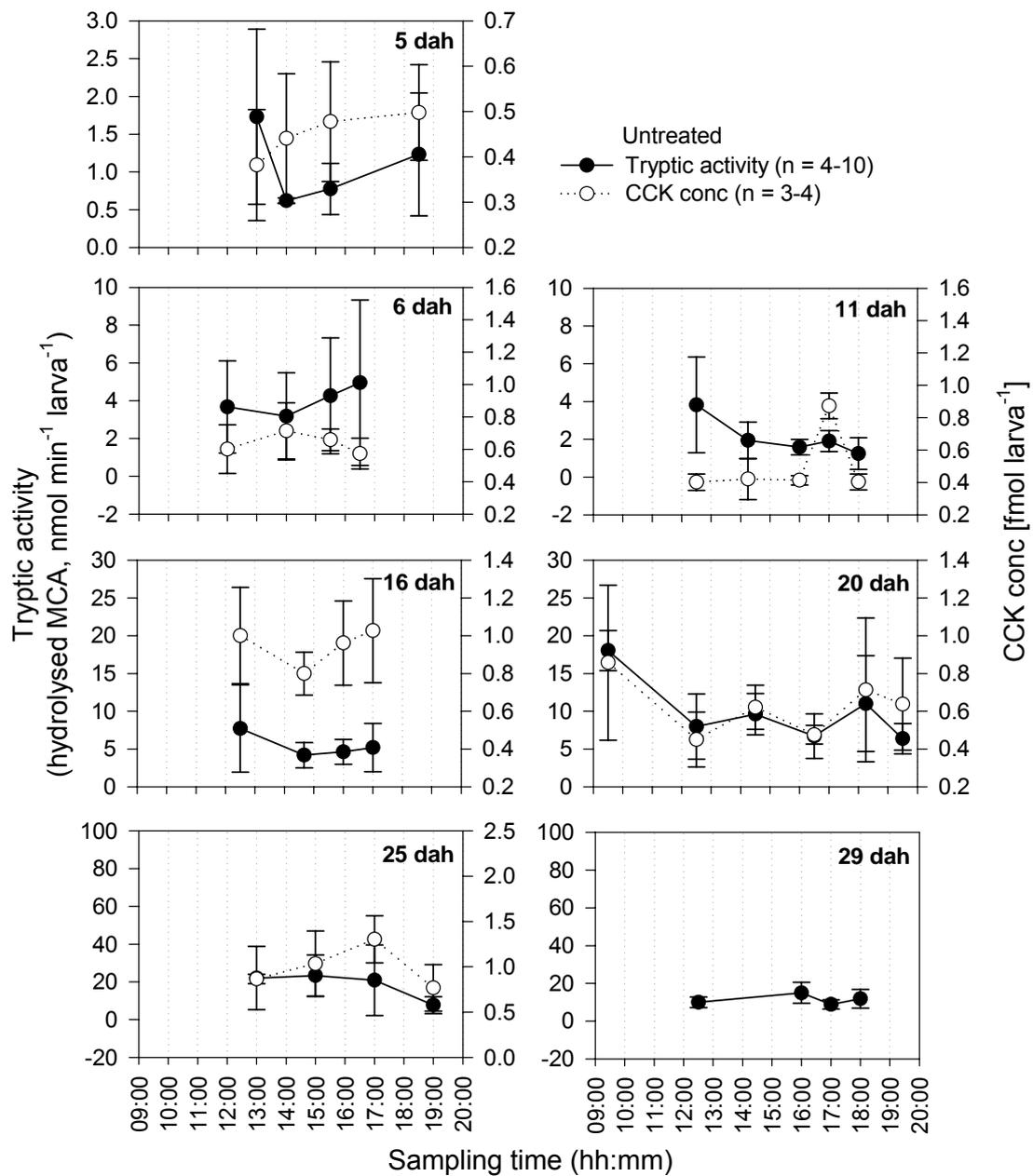
Fig. 39: Untreated herring larvae: tryptic activity and CCK content in relation to the sampling time at 5, 6, 11, 16, 20, 25 and 29 dah (the CCK content was not determined in larvae aged 29 dah). Data are given as means (symbols) with standard deviations (bars). Note the differing scaling of the y-axes on each day. n = number of individual measurements per mean
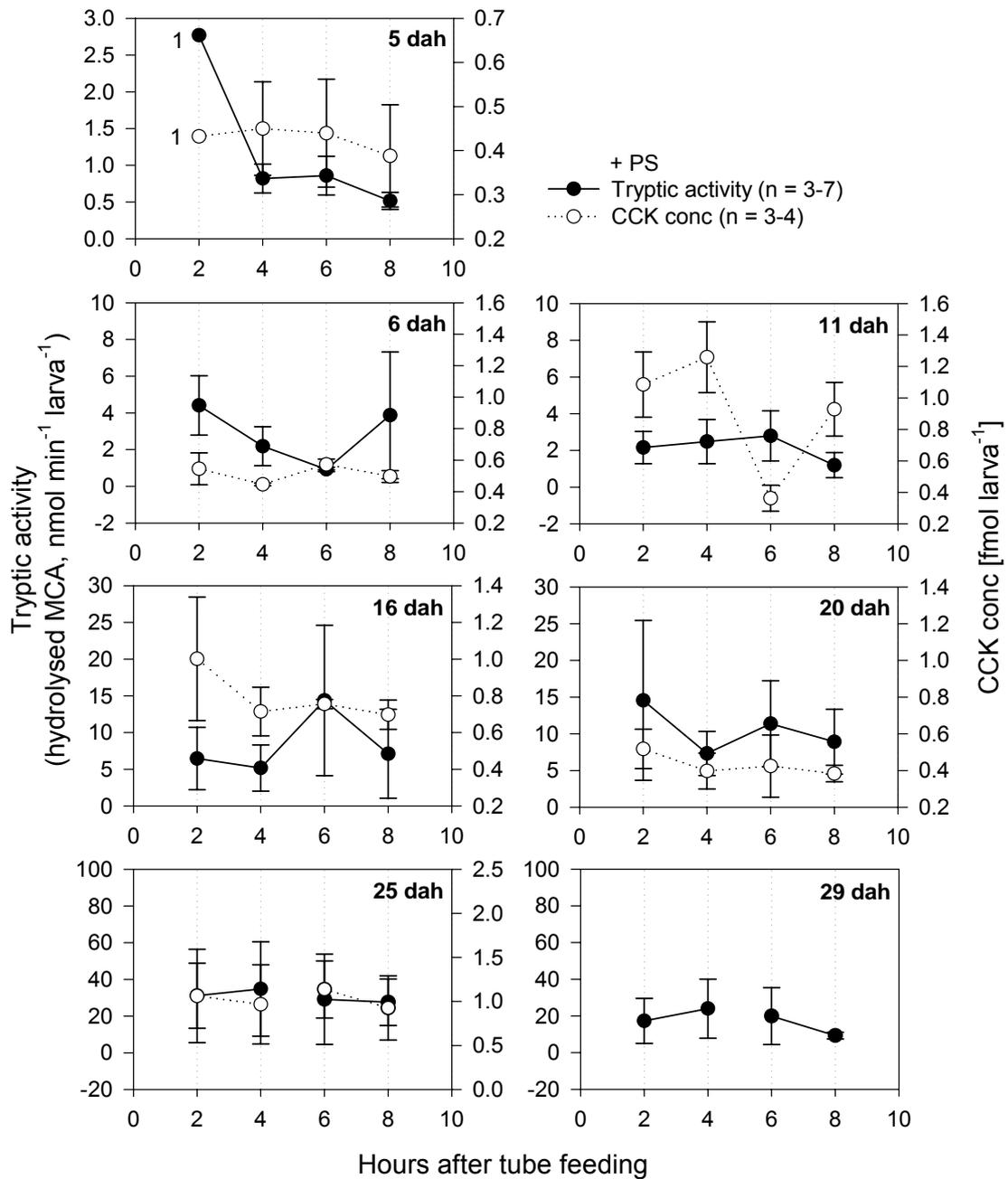
Fig. 40: Herring larvae micro-injected with physiological saline (PS): tryptic activity and CCK content in relation to the incubation period after treatment at 5, 6, 11, 16, 20, 25 and 29 dah (the CCK content was not determined in larvae aged 29 dah). Data are given as means (symbols) with standard deviations (bars). Means that are not connected with a line represent not successive sampling times (e.g. a larva 8 h after treatment can have been micro-injected and sampled earlier than one 6 h after treatment). Note the differing scaling of the y-axes on each day. n = number of individual measurements per mean
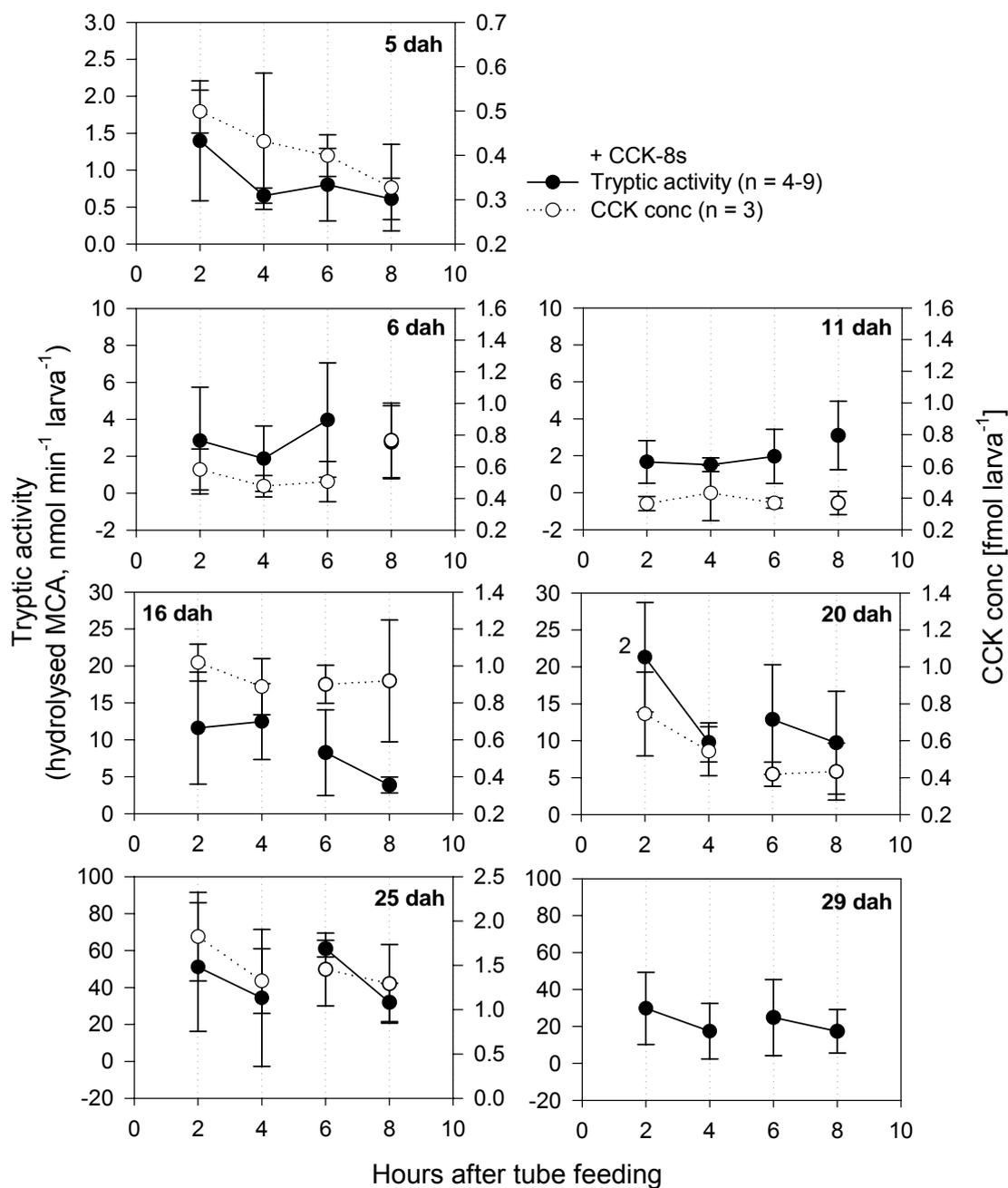
Fig. 41: Herring larvae treated with the hormone cholecystokinin (CCK-8s): tryptic activity and CCK content in relation to the incubation period after application of a micro-injection at 5, 6, 11, 16, 20, 25 and 29 dah (the CCK content was not determined in larvae aged 29 dah). Data are given as means (symbols) with standard deviations (bars). Means that are not connected with a line represent not successive sampling times (e.g. a larva 8 h after treatment can have been micro-injected and sampled earlier than one 6 h after treatment). Note the differing scaling of the y-axes on each day. n = number of individual measurements per mean
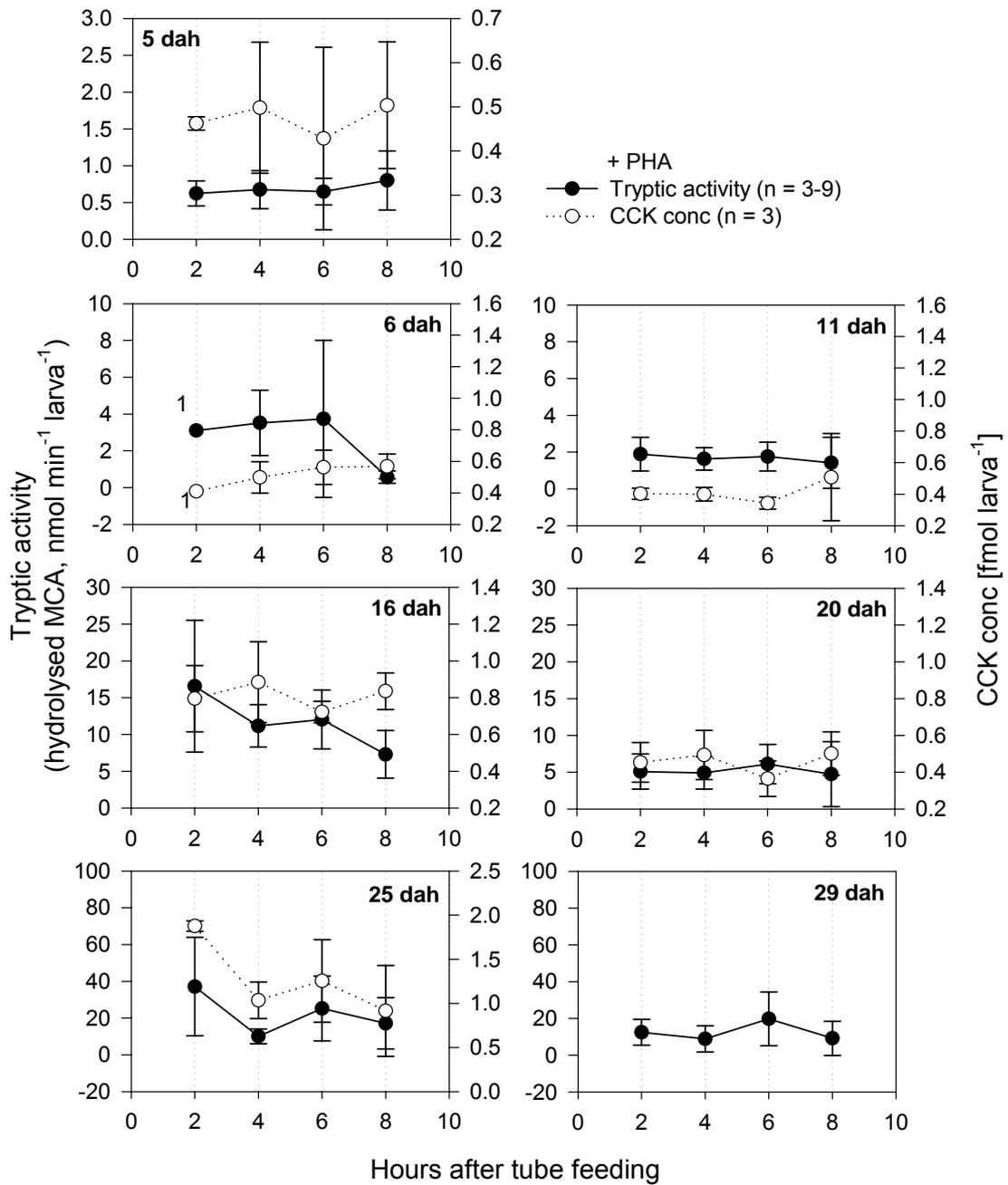
Fig. 42: Herring larvae treated with the plant protein phytohemagglutinin (PHA): tryptic activity and CCK content in relation to the incubation period after application of a micro-injection at 5, 6, 11, 16, 20, 25 and 29 dah (the CCK content was not determined in larvae aged 29 dah). Data are given as means (symbols) with standard deviations (bars). Note the differing scaling of the y-axes on each day. n = number of individual measurements per mean

Fig. 43 compares the CCK content between untreated larvae and those micro-injected with physiological saline, at different days during the experimental period and different sampling times. CCK concentration was significantly different between untreated and treated controls at all four sampling times only on day 11 after hatching, indicating a handling or technical problem.
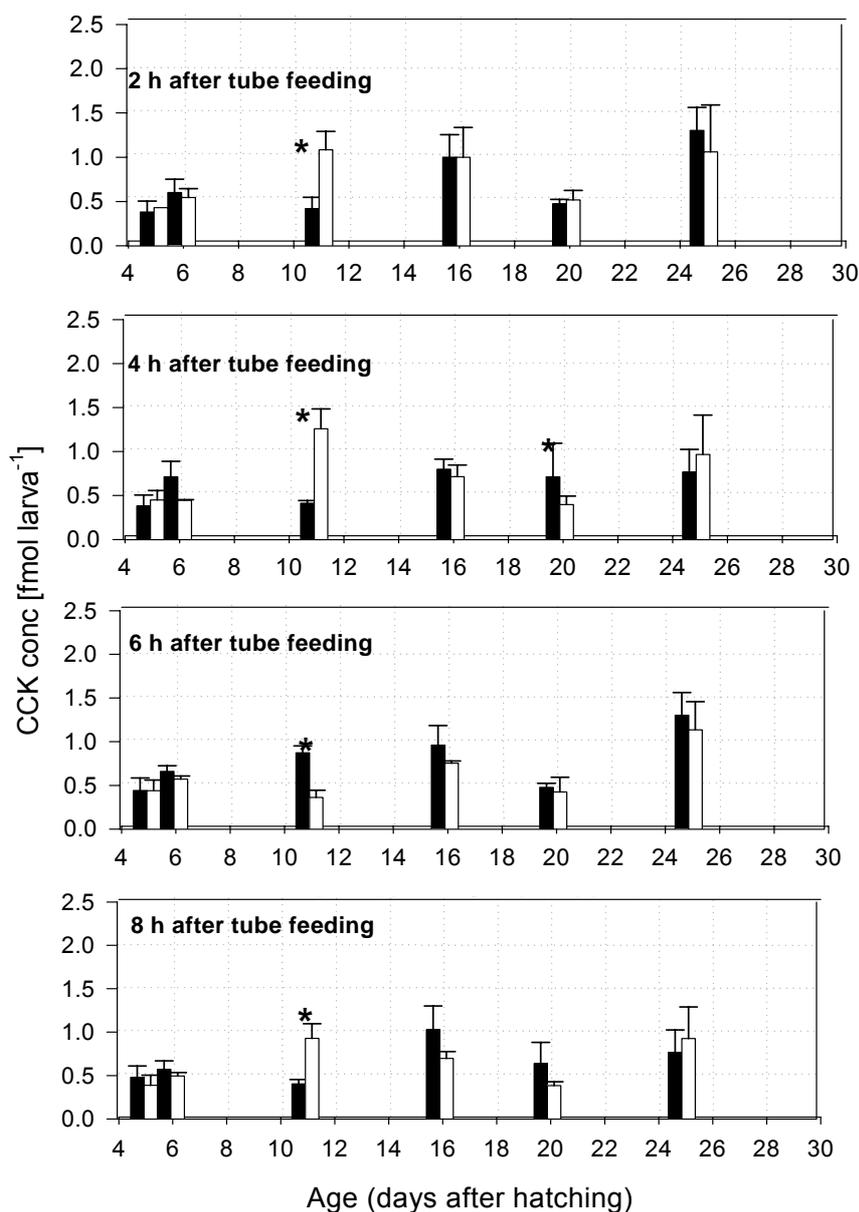

Fig. 43: Comparison of CCK content between herring larvae that were not treated (■) and those micro-injected with physiological saline (PS) (□) at selected days during the experimental period. Data are given as means (symbols) with standard deviations (bars). The time after treatment marking each graph refers to the incubation period of the PS-group, while untreated larvae were sampled at the respective time of the day. Pairs with significant differences are marked by an asterisk. n = 3-4 individual measurements per mean

Fig. 44 compares the tryptic activity between untreated and treated controls (PS group), at different days during the experimental period and different sampling times. Significant differences were found between tryptic activity levels of the two groups at different days and different times, mostly being higher in the PS than in the untreated group. Similarly to the results from halibut larvae, the observed differences indicate that further comparisons of treated groups should be made only with the PS group as a control.
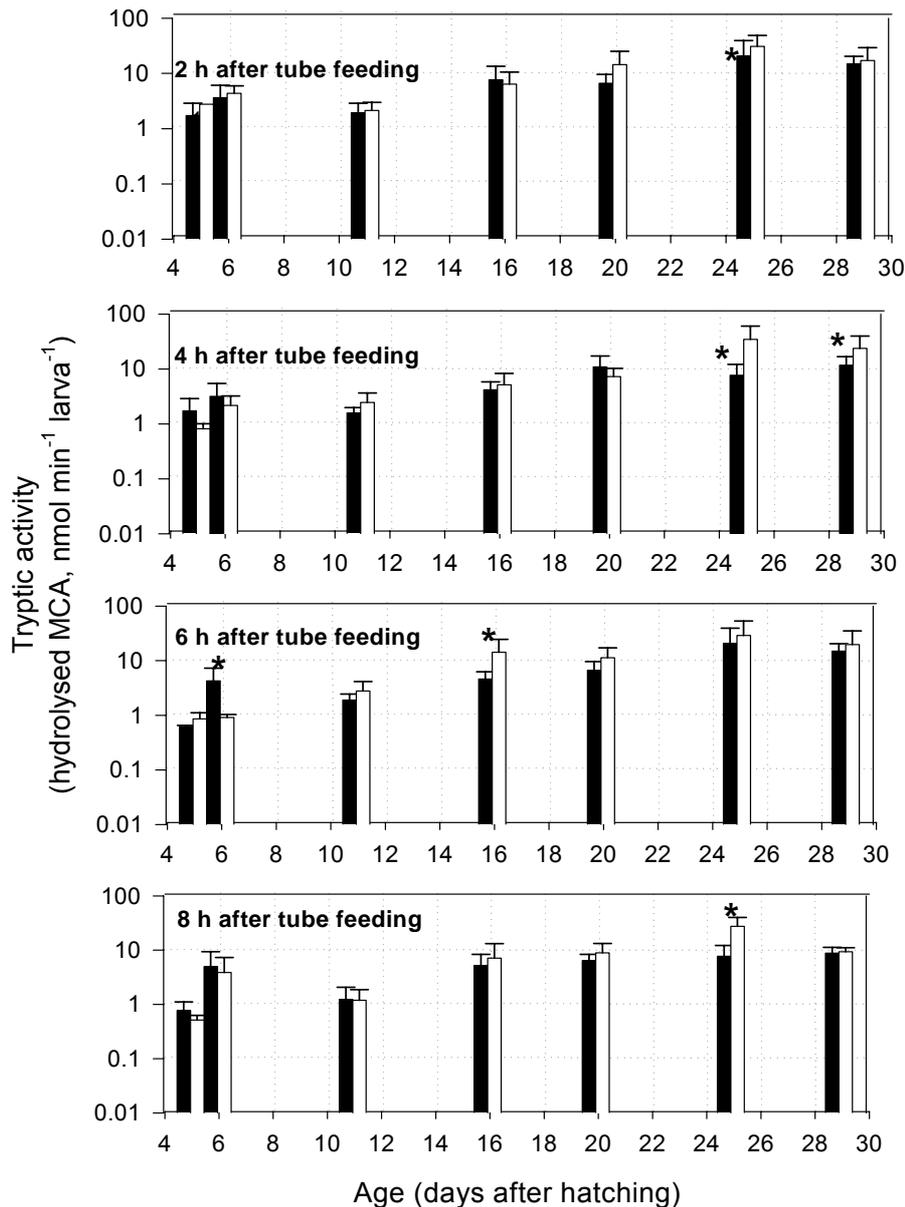

Fig. 44: Comparison of tryptic activity between herring larvae that were not treated (■) and those micro-injected with physiological saline (PS) (□) at selected days during the experimental period. Data are given as means (symbols) with standard deviations (bars). The time after treatment marking each graph refers to the incubation period of the PS-group, while untreated larvae were sampled at the respective time of the day. Pairs with significant differences are marked by an asterisk. Note the logarithmic scaling of the y-axis. n = 3-10 individual measurements per mean

In Fig. 45 and 46 the CCK content and tryptic activity, respectively of herring larvae treated with CCK-8s are shown in comparison to that of treated controls (PS group), in relation to the incubation period after application of a single micro-injection on selected days during the experimental period. Herring larvae treated with CCK-8s at 5, 6, 11, 16, 20 and 25 dah showed the highest CCK concentrations 2 hours after micro-injection (Fig. 45). When compared to the control group micro-injected with physiological saline, the CCK-8s group showed more or less the same development with incubation period, but with higher CCK levels at 16, 20 and 25 dah. When compared to the PS controls, significant higher values were also found in tryptic activity of the CCK-8s group at 6, 16 and 25 dah (Fig. 46). However, it was not possible to distinguish a certain incubation range necessary to achieve higher enzymatic activities by the applied CCK-8s. Thus, a direct effect of externally applied CCK-8s on larval tryptic activity was not evident.

In Fig. 47 the CCK content of herring larvae treated with PHA is compared to that of treated controls (PS group), and shown in relation to the incubation period after application of a single micro-injection on selected days during the experimental period. In contrast to the CCK-8s group, in larvae treated with PHA the highest CCK concentrations were recorded 8 hours after micro-injection, with exception of day 25 after hatching. The development of the CCK content in the PHA group with incubation period was opposite to that in the PS group, at almost every treatment day: when CCK content in the PS group was declining, secretion of CCK was observed in larvae micro-injected with PHA solution (in herring larvae the differentiation between production and secretion is possible, since larval samples were analysed without heads, and the contribution of brain CCK can be excluded).

In Fig. 48 tryptic activity is compared between the PS controls and the PHA group, and is shown in relation to the incubation period after application of a single micro-injection on every treatment day. On day 5 and 6 after hatching herring larvae still having yolk reserves showed opposite trends in the daily development of tryptic activity between PS and PHA group. During the following days though, tryptic activity was not evidently elevated due to the treatment with PHA.
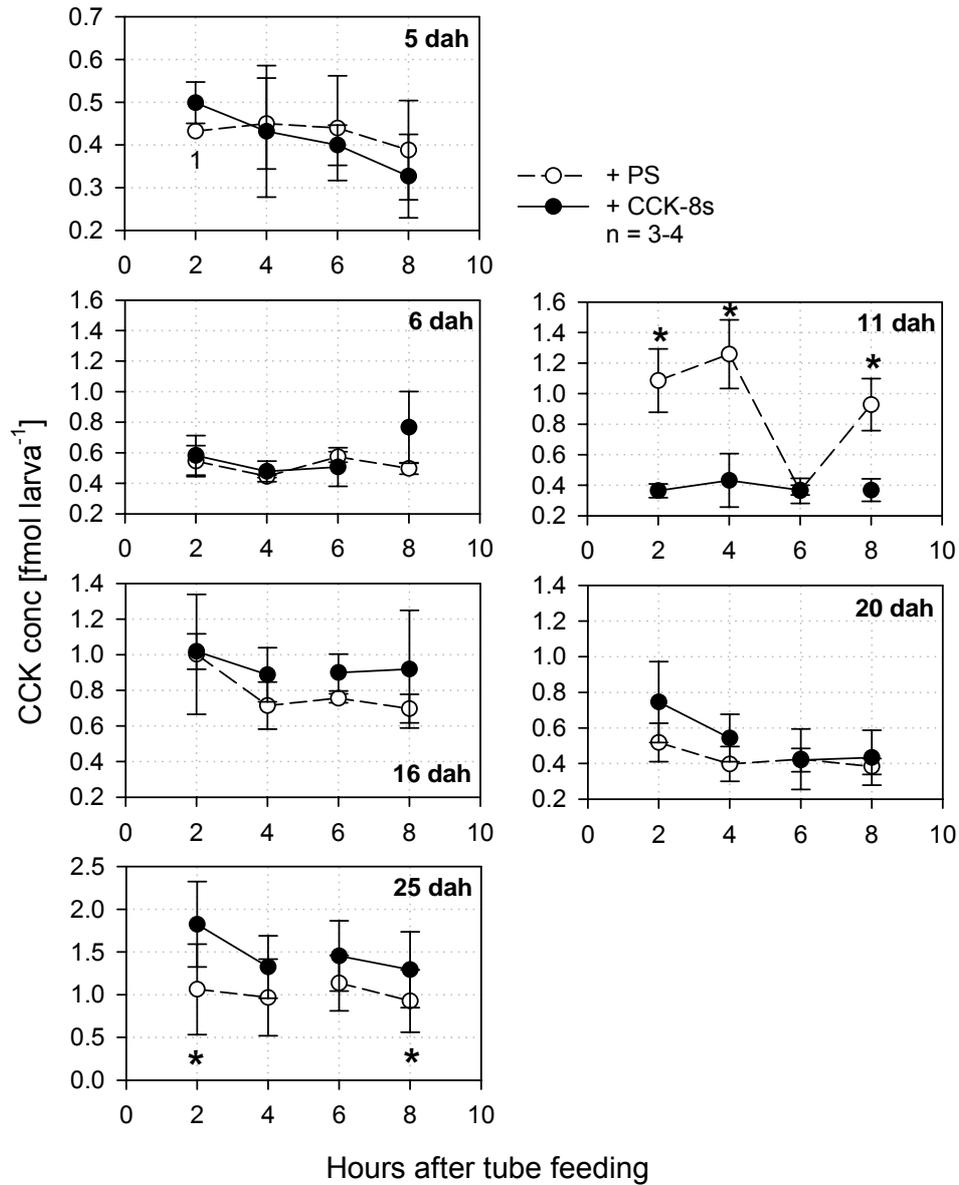
Fig. 45: Comparison of CCK content between herring larvae that were micro-injected only with physiological saline (PS) and those treated with the hormone cholecystokinin (CCK-8s) at 5, 6, 11, 16, 20 and 25 dah. Data are related to the incubation period after treatment and are given as means (symbols) with standard deviations (bars). Means that are not connected with a line represent not successive sampling times (e.g. a larva 8 h after treatment can have been micro-injected and sampled earlier than one 6 h after treatment). Pairs with significant differences are marked by an asterisk. Note the differing scaling of the y-axes on each day. n = number of individual measurements per mean
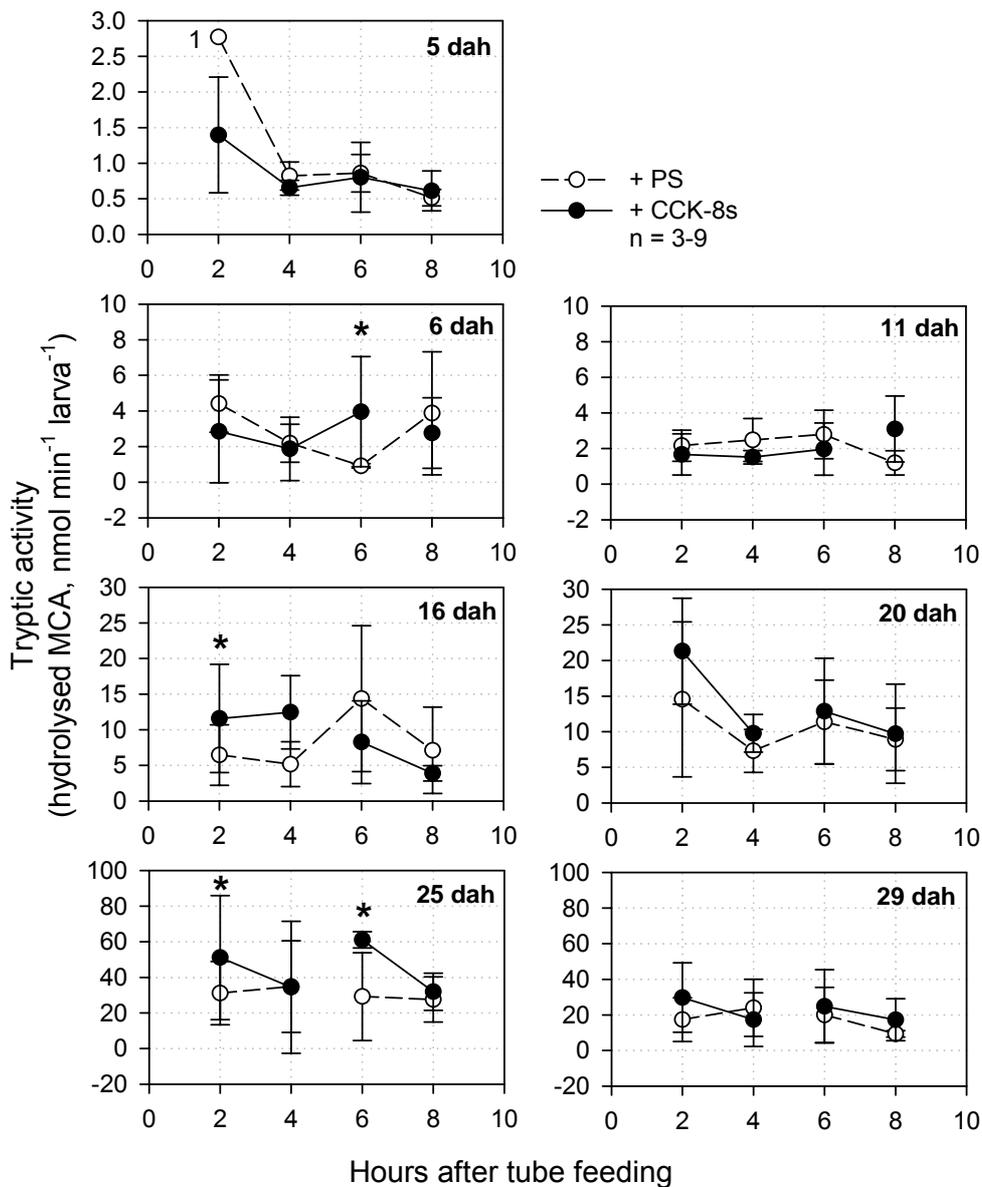
Fig. 46: Comparison of tryptic activity between herring larvae that were micro-injected only with physiological saline (PS) and those treated with the hormone cholecystokinin (CCK-8s) at 5, 6, 11, 16, 20, 25 and 29 dah. Data are related to the incubation period after treatment and are given as means (symbols) with standard deviations (bars). Means that are not connected with a line represent not successive sampling times (e.g. a larva 8 h after treatment can have been micro-injected and sampled earlier than one 6 h after treatment). Pairs with significant differences are marked by an asterisk. Note the differing scaling of the y-axes on each day. n = number of individual measurements per mean
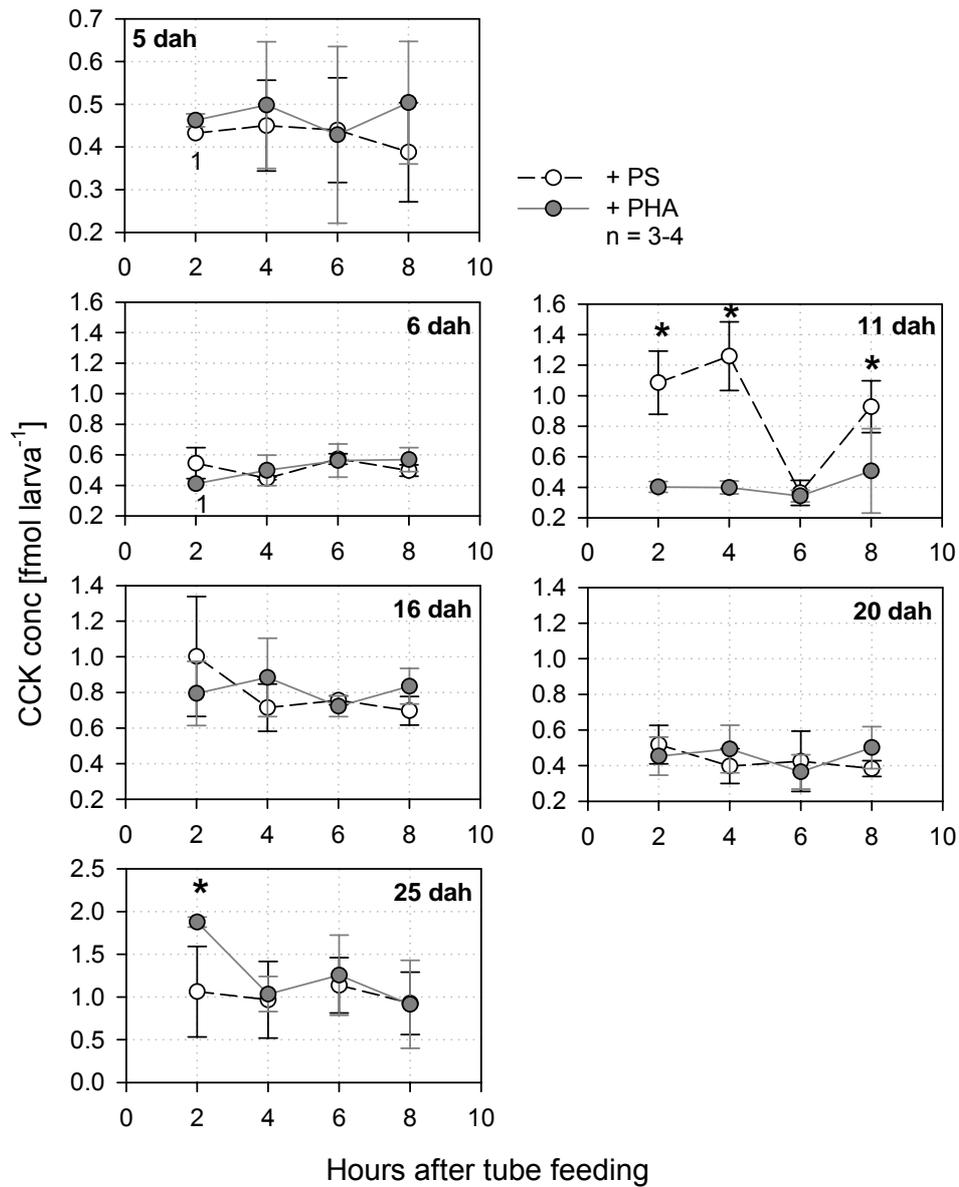
Fig. 47: Comparison of CCK content between herring larvae that were micro-injected only with physiological saline (PS) and those treated with the plant protein phytohemagglutinin (PHA) at 5, 6, 11, 16, 20 and 25 dah. Data are related to the incubation period after treatment and are given as means (symbols) with standard deviations (bars). Means that are not connected with a line represent not successive sampling times (e.g. a larva 8 h after treatment can have been micro-injected and sampled earlier than one 6 h after treatment). Pairs with significant differences are marked by an asterisk. Note the differing scaling of the y-axes on each day. n = number of individual measurements per mean
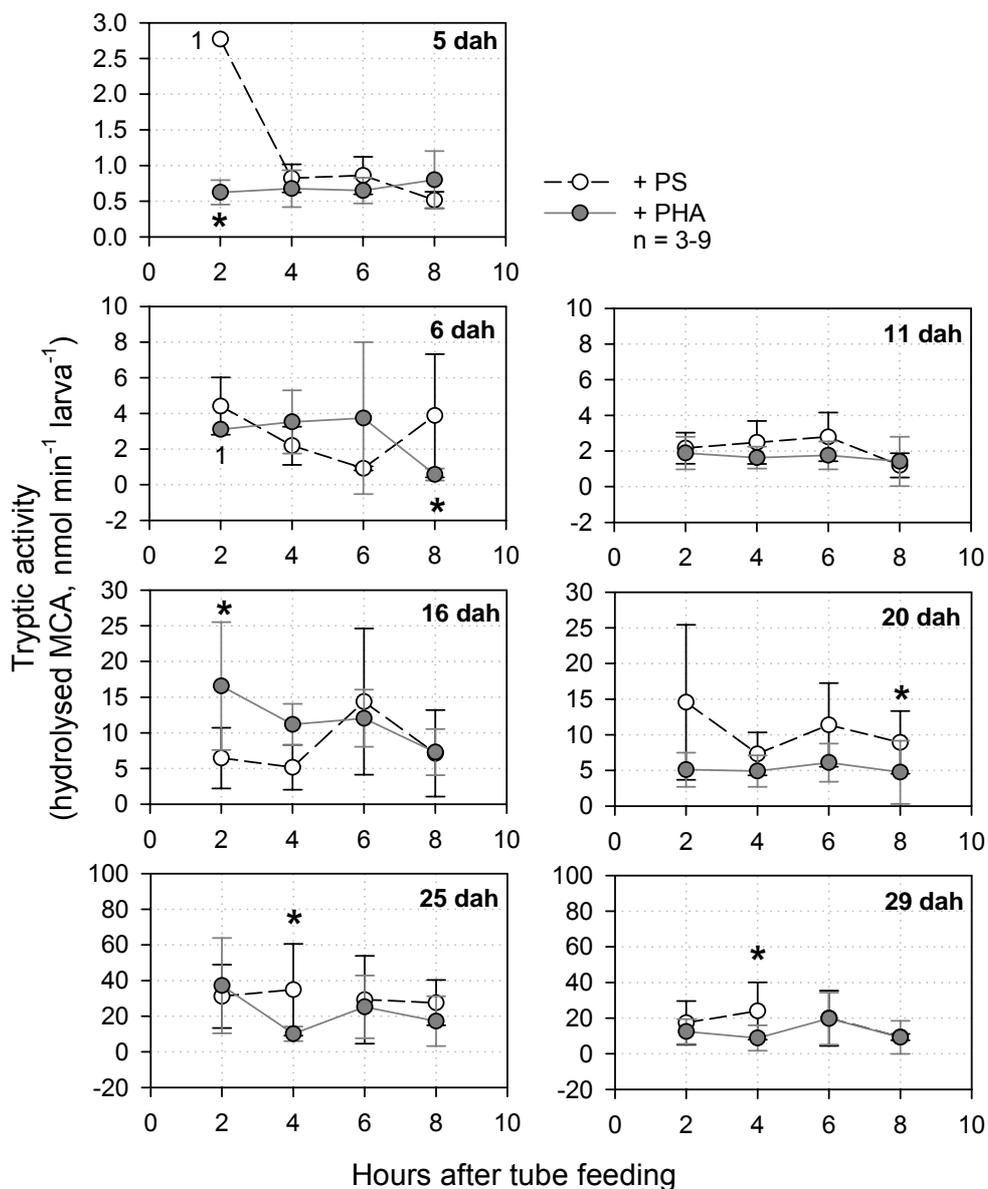
Fig. 48: Comparison of tryptic activity between herring larvae that were micro-injected only with physiological saline (PS) and those treated with the plant protein phytohemagglutinin (PHA) at 5, 6, 11, 16, 20, 25 and 29 dah. Data are related to the incubation period after treatment and are given as means (symbols) with standard deviations (bars). Means that are not connected with a line represent not successive sampling times (e.g. a larva 8 h after treatment can have been micro-injected and sampled earlier than one 6 h after treatment). Pairs with significant differences are marked by an asterisk. Note the differing scaling of the y-axes on each day. n = number of individual measurements per mean

3.5 CCK content and tryptic activity in cod larvae

As in herring, the daily sampling of cod larvae revealed opposite developments of the CCK content and tryptic activity with age (Fig. 49). This interaction was obvious throughout the experiment in untreated larvae and those immersed daily in a solution with 0.2 µM CCK-8s. In the group treated with the lower CCK-8s concentration (0.02 µM), opposite developments of CCK level and tryptic activity were observed until 10 dah, but during the following days trends in both parameters were the same.
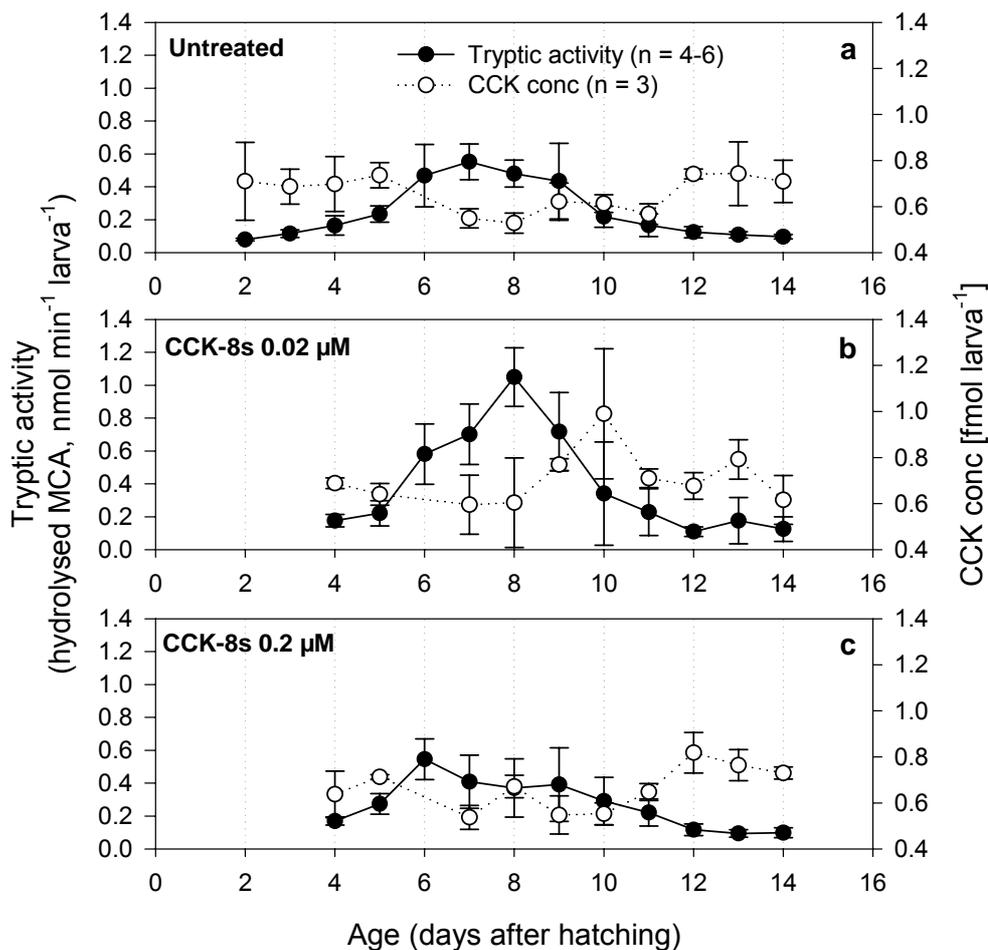

Fig. 49: Development of tryptic activity and CCK content of cod larvae during the experimental period: a) untreated controls, b) and c) larvae treated with the hormone cholecystokinin (CCK-8s) by daily immersions in a 0.02 µM and 0.2 µM solution, respectively. Larval samples were taken at 12:30 h, corresponding to the sampling time after treatment. Data are given as means (symbols) with standard deviations (bars). n = number of individual measurements per mean

CCK as well as tryptic activity were already detectable in embryonic stages (Fig. 50). While the CCK content in embryos increased towards hatching (Fig. 50a), tryptic activity showed a low depot before hatching, which reflected the starting level in newly hatched larvae (Fig.

50b). After hatching, tryptic activity in fed larvae increased until 7 dah to 0.55 ± 0.11 nmol/min (n = 6). Thereafter, enzyme activity decreased until the end of the experiment at 14 dah, reaching 0.10 ± 0.01 nmol/min (n = 6), which was almost the same low level as at hatching (0.08 ± 0.01 nmol/min, n = 6). The highest increment in tryptic activity was observed between 5 and 6 dah; the highest decline between 9 and 10 dah. The same development of tryptic activity was observed in larvae that were not first fed, but with significantly lower values from 7 dah onwards. In regularly fed larvae the development of the CCK concentration with age was observed to depend on the tryptic activity values. While a higher CCK concentration was recorded from hatching until 5 dah (0.74 ± 0.05 fmol, n = 3), thereafter the CCK content declined and remained in lower levels as long as tryptic activity in fed larvae was significantly higher than that in unfed individuals. On day 12 after hatching, when tryptic activity had fallen to 0.12 ± 0.03 nmol/min (n = 5), the CCK content increased again (0.74 ± 0.02 fmol, n = 3) to the initial level that had been measured in newly hatched larvae.

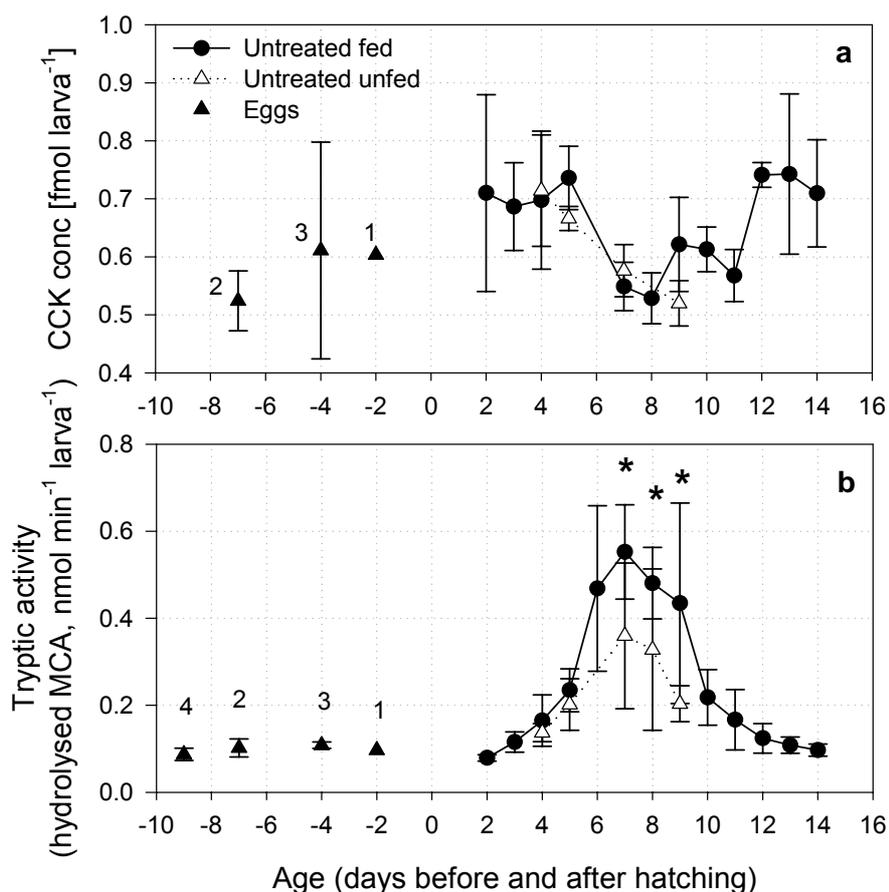Fig. 50: Comparison of CCK content (a) and tryptic activity (b) between untreated cod embryonic stages, fed larval controls and larvae that were not first fed. The sampling time was at 12:30 h. Data are given as means (symbols) with standard deviations (bars). Pairs with significant differences are marked by an asterisk. The number of individual larval measurements (n) was 3 for CCK content and 5-6 for tryptic activity

To summarise, CCK increased when tryptic activity in regularly fed larvae reached starvation levels, and remained on a base level when trypsin secretion was sufficiently high. In contrast to fed individuals, cod larvae that were not offered any food, did not seem to react to the declining tryptic activity on day 9 after hatching.

The direct comparisons of CCK content and tryptic activity between the PS group and larvae treated by daily immersions with two different concentrations of CCK-8s are shown in Fig. 51 and 52, respectively. When compared to untreated controls, cod larvae treated with 0.02 µM CCK-8s showed higher CCK concentrations from 7 (no data point at 6 dah available) to11 dah (Fig. 51a), and higher tryptic activities from 6 to 11 dah (Fig. 52a). Trends due to the respective developmental stage were still dominant, but there was evidence for an effect initiated by the CCK-8s treatment on CCK content and tryptic activity of cod larvae older than 6 dah. In contrast, larvae treated with the higher CCK-8s concentration (0.2 µM CCK-8s solution) showed no great differences either in CCK content, nor in tryptic activity when compared with the untreated group (Fig. 51b and 52b), indicating an overdose of CCK-8s.

Fig. 51: Comparison of CCK content between untreated cod larvae and those treated with the hormone cholecystokinin (CCK-8s) by daily immersions in a 0.02 µM (a) or 0.2 µM (b) solution. Larval samples were taken at 12:30 h, corresponding to the time after treatment. Data are given as means (symbols) with standard deviations (bars). Pairs with significant differences are marked by an asterisk. n = number of individual measurements per mean
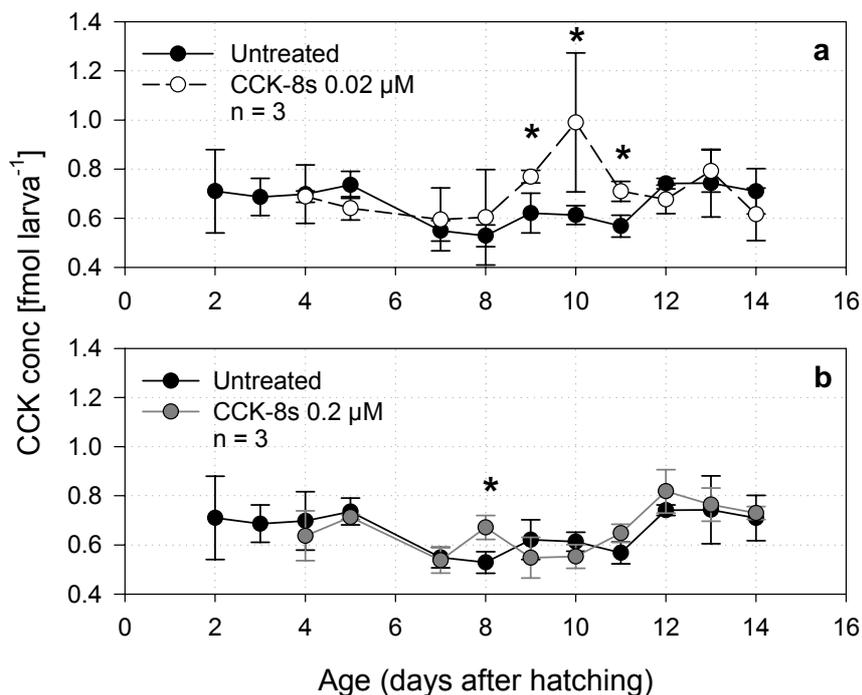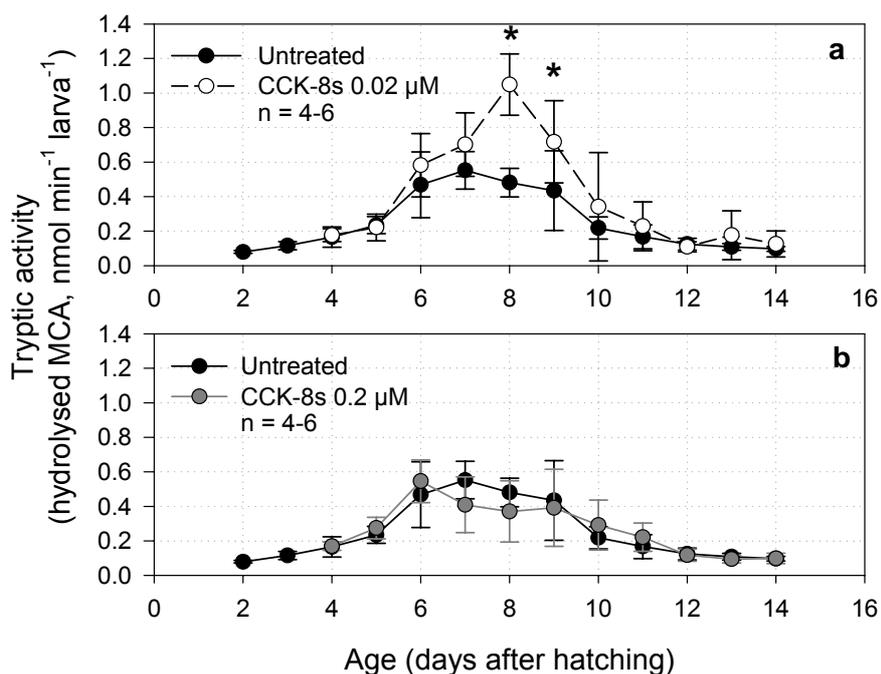
Fig. 52: Comparison of tryptic activity between untreated cod larvae and those treated with the hormone cholecystokinin (CCK-8s) by daily immersions in a 0.02 µM (a) or 0.2 µM (b) solution. Larval samples were taken at 12:30 h, corresponding to the time after treatment. Data are given as means (symbols) with standard deviations (bars). Pairs with significant differences are marked by an asterisk. n = number of individual measurements per mean

On some days cod larvae were sampled just before and immediately after the immersion treatment. Fig. 53 and 54 show the CCK content and tryptic activity, respectively of untreated controls and treated individuals in relation to the larval age and the sampling time. Although diurnal cycles already influence hormonal levels and enzyme activities, in the comparative plots of samples taken at 09:30 and at 12:30 h, corresponding to the time just before and after treatment, significant differences in CCK concentration as well as in tryptic activity were found only in the 0.02 µM CCK-8s group. This observation enhances the above results that only the lower CCK-8s concentration achieved a manipulative effect. CCK and tryptic activity values before treatment were comparable in all groups, suggesting that CCK in long-term treated larvae was only elevated immediately after treatment and not during the whole day.
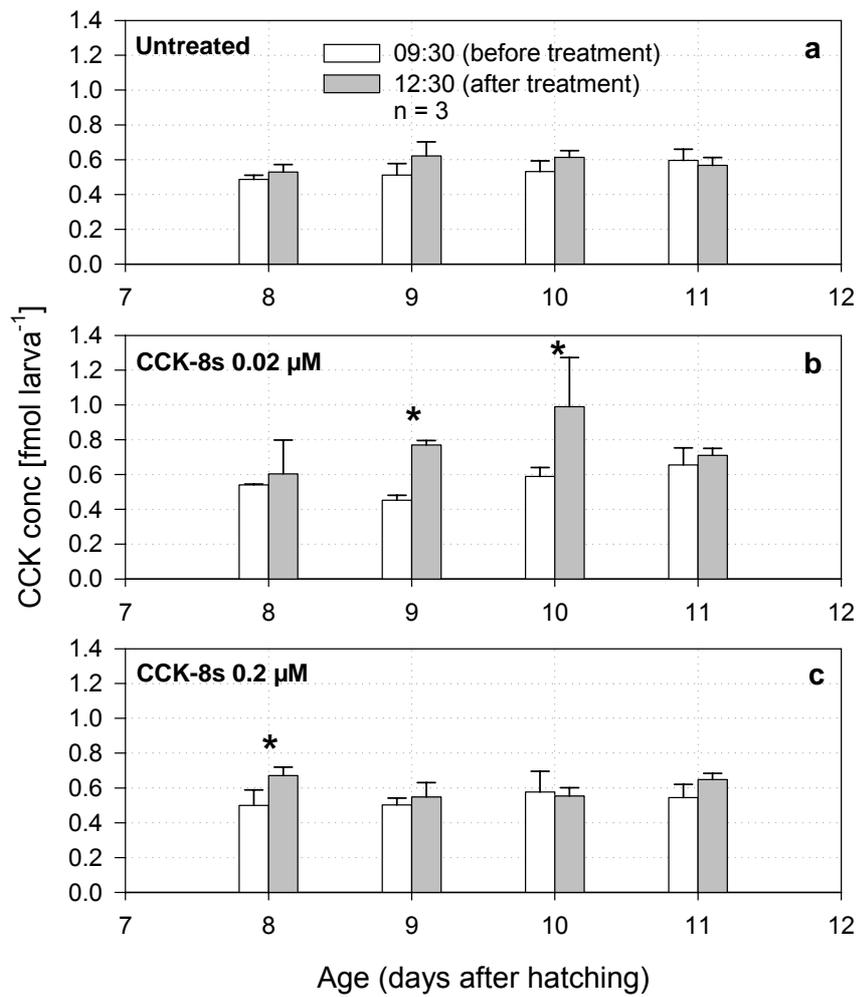
Fig. 53: Comparison of CCK content between two daily sampling times: a) untreated controls sampled at 09:30 and 12:30 h, b) and c) larvae treated with the hormone cholecystokinin (CCK-8s) by daily immersions in a 0.02 μM and 0.2 μM solution, respectively and sampled before and after treatment. Data are given as means (symbols) with standard deviations (bars). Pairs with significant differences are marked by an asterisk. n = number of individual measurements per mean
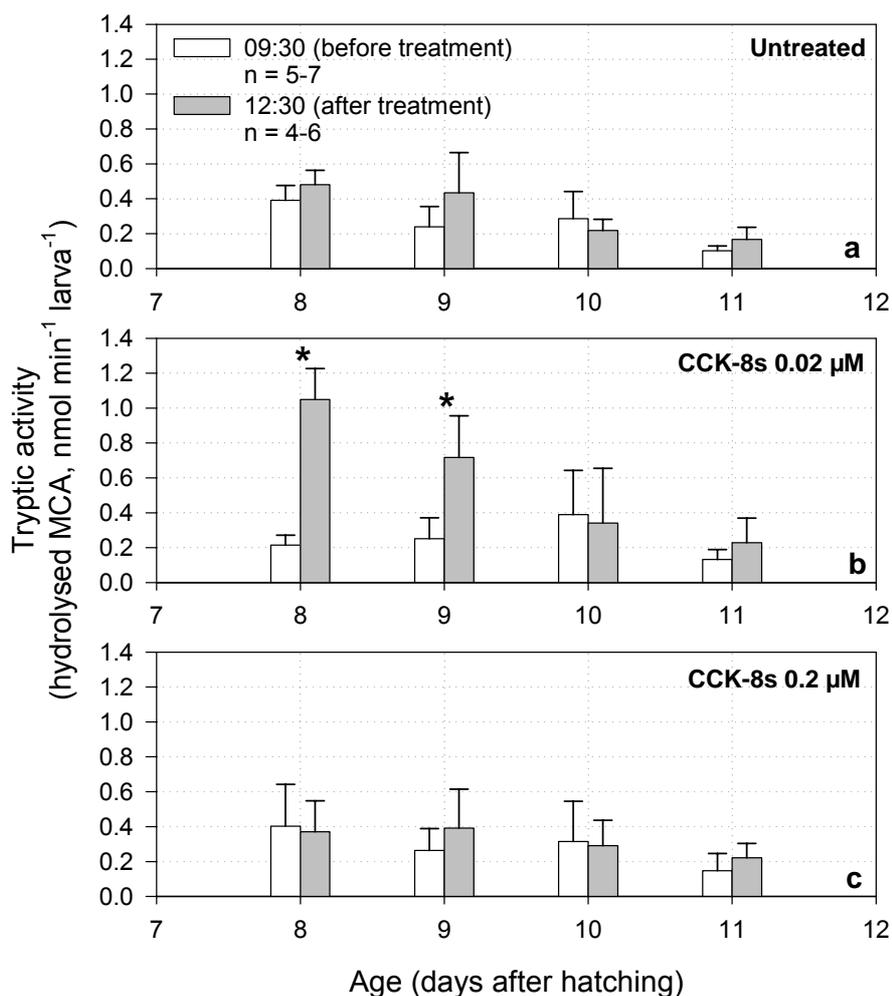
Fig. 54: Comparison of tryptic activity between two daily sampling times: a) untreated controls sampled at 09:30 and 12:30 h, b) and c) larvae treated with the hormone cholecystokinin (CCK-8s) by daily immersions in a 0.02 µM and 0.2 µM solution, respectively and sampled before and after treatment. Data are given as means (symbols) with standard deviations (bars). Pairs with significant differences are marked by an asterisk. n = number of individual measurements per mean

4 Discussion

The present work focused on the study of the gastrointestinal hormone cholecystokinin (CCK) and the activity of the pancreatic enzyme trypsin in larval stages of marine fish species. A feedback relationship between CCK secretion and tryptic activity is known in mammals and adult fishes, but in fish larvae from hatching to metamorphosis the mechanisms involving these two parameters are still unknown.

In order to test whether it is possible to manipulate the CCK level and consequently the tryptic enzyme activity in the gut of marine fish larvae, a direct way through treatment with the physiological hormone CCK-8s and an indirect way through administration of the plant protein phytohemagglutinin (PHA) as a CCK releasing stimulus, were chosen. Additionally, two different applications were performed. Larvae of Atlantic halibut (*Hippoglossus hippoglossus*) and Atlantic herring (*Clupea harengus*) were short-term treated with single *in vivo* micro-injections allowing the direct administration of the test solutions into the larval gut. The test solutions consisted either only of physiological saline as a treatment control, or contained CCK-8s in a concentration of 0.1143 ng per mg wet weight or PHA in a concentration of 0.003% of the larval wet body weight. The larvae were treated with a single micro-injection at selected days and sampled at 2, 4, 6 and 8 hours after treatment. A long-term treatment was performed on larvae of Baltic cod (*Gadus morhua*), which were immersed daily for 1½ hour in CCK-8s solutions of two different concentrations (PHA was not tested here) over a period of 11 days. The amount of CCK-8s that was ingested by the treated cod larvae through drinking was estimated at a maximum of 0.1 pg/larva after treatment with the lower concentrated solution (0.02 µM CCK-8s), and at a maximum of 1 pg/larva after treatment with the higher concentrated solution (0.2 µM CCK-8s).

Data of standard length, dry weight, CCK concentration and tryptic enzyme activity were obtained from each sampled individual. For the chemical analysis of CCK level (by radioimmunoassay) and tryptic activity (by a fluorometric method) on individual larvae, a combined preparation protocol was tested, allowing the use of small sample amounts. The radioimmunoassay for the measurement of CCK concentrations (CCK-RIA) was successfully performed the first time in the IFM-GEOMAR.

4.1 Advantages and constraints of the micro-tube feeding procedure

Studies on nutrient composition are especially difficult with diets for larval fish, which typically feed on particle sizes between 50 and 150 µm (Rønnestad *et al.*, 2001). The micro-tube feeding method used in these experimental series enables a controlled delivery

of test diets to larvae immediately after mouth opening. After practical experience with the involved handling, a critical consideration can be useful for future approaches.

Larvae of halibut and herring tolerated well the stress imposed by handling for the micro-injection method. Mortality due to handling was low, with 6% and 24.9%, respectively. The concentration of the anaesthetic (30 µg/ml MS-222) was chosen as a compromise between (a) the anaesthetic effect on the larvae to allow handling and injection, (b) the importance to maintain opercular movements to ensure survival, and (c) a rapid recovery to ensure proper physiological function of the digestive system (Rust *et al.,* 1993; Rønnestad *et al.,* 2000a). Considering the fact that most of the halibut larvae were "gapers", characterised by an overall weak condition, it can be concluded that the stress imposed by the method is rather moderate. On the other hand, initial tests with larvae of Atlantic cod (10-12 and 23 dah) had to be abandoned, because of the high mortality of up to 76.4% after the micro-injections. Similar results were obtained by other researchers working at that time on cod at the same facilities (personal communications). Rønnestad *et al.* (2001) summarised the survival results of different species in different developmental stages used in previous micro-injection studies, and pointed out that stress-related effects depend mainly on the developmental stage of the species and on the quality of the larval batch. The results of the present experiments indicate that morphometric characteristics, such as the size of the larva at the time of mouth opening and the form of the larval gut are major contributing factors.

Tests with repeated injections on the same herring larvae were successful, indicating that a repeated treatment might also be possible (a longer period with more than two consecutive injection days should though still be tested). Nevertheless, in order to use this method for long-term treatments a big number of larvae would have to be injected daily, which is a time-consuming procedure. In the present experiments at least 72 larvae were injected on every treatment day: 6 larvae for each of the three test-solutions and of the four incubation periods. While the actual injection is done within a few seconds, the whole handling per individual from the time the larva is removed from the bucket and positioned under the microscope, until it is placed in the incubation well, can take up to 3 minutes. A very time consuming procedure is the capillary exchange between different test-solutions, which has to be performed often, depending on the incubation periods and treatment groups. Considering also the fact that the injections took place in a 10°C cold room, which forces one to make more breaks in order to warm up again (the fingers have to be warm and able of carrying out such a precise work), makes the daily work effort obvious. Thus, the method is more suitable for short-term studies with smaller sampling groups, and is not an easy option for monitoring the effects of a multiple injected diet over a longer period.

According to Rønnestad *et al.* (2001), careful handling during the micro-injection procedure does not affect the larval metabolism. However, in the present study peristaltic movements were observed in the gut of herring larvae immediately after the injections, also reported by Koven *et al.* (2002) and Morais *et al.* (2005), supporting the observations of Rust *et al.* (1993) that the introduction of the capillary into the gut stimulates processes involved in digestion itself. Morais *et al.* (2005) reported that the observed contractions were of the standing type (local, unpropagated smooth muscle contractions), responsible for mixing the gastrointestinal content. The comparison of CCK content and tryptic activity in untreated larvae and those micro-injected with physiological saline (PS) as a treatment control, also showed differences between the two groups. Lower values observed in the PS group could have been due to an initial washing-out effect of endogenous CCK or trypsin from the gut, through the introduced volume of the applied solution, although 2 hours after treatment seems long enough for a reestablishment of normal metabolic conditions. In contrast, values of CCK and tryptic activity higher in the PS group than in untreated larvae give evidence for a simulation of feed intake through gut distension, caused by the injection volume.

According to Rust *et al.* (1993) the tube-feeding method can also be used to test slurries of small particulate diets, but concluding from the experience in this work such an approach is not without limitations. The concentration and thus the viscosity of the test solution in combination with the capillary diameter and the intended injection volume can be a restricting factor, since too thick flowing liquids can not be loaded properly. Especially at lower injection volumes it is advisable to use coloured solutions to ensure the delivery control, if additional effects of the dye used can be excluded. The control via visible slight gut extension due to such a small volume as 9.2 nl used here, was not always given, depending on the previous relaxation and distension of the larval digestive tract.

Another essential point is that the method allows the delivery of a specific concentration, but is no evidence for the full absorption of the tested nutrient. Without additional measurements in samples from the incubation medium, it is not possible to detect whether parts of the injected solution get lost through vomit or unabsorbed postprandial excretion. Thus, the data reflect the relative amount retained in the body, and an underestimation of the total absorbed amount from the gut has to be considered (Rønnestad *et al.*, 2001).

Nevertheless, while stress imposed by the technique or even the technique itself may have an impact on absolute values, relative differences among the solutions tested within these trials ought to reflect real differences. Yet, the discussed limitations may explain higher deviations among the presented results. In general, it can be concluded that in studies like this it is necessary that the control group represents larvae treated with physiological

saline, in the same manner as the experimental groups. A comparison of treated larvae with an untreated group as the only control would not be correct and might lead to false conclusions due to underestimation of the treatment effect.

4.2   Assessment of the immersion handling for long-term treatments of fish larvae

Hypo-osmoregulation is an essential balance mechanism for organisms living in the hyperosmotic marine environment. In adult fish, seawater is being absorbed through the body surface, the gills and the gut, and excretion of excess salts occurs by chloride cells and the kidney. Since larval osmoregulation organs are in a primordial stage, and fish larvae have a greater body surface to mass ratio than adults, a high body surface permeability would be expected. However, against expectation, larvae are less permeable than adults (Tytler and Bell, 1989), and permeability increases towards metamorphosis (Tytler *et al.*, 1993). In fact, drinking activity has been confirmed to be the major contributing mechanism for volume and ionic regulation of body fluids in marine fish larvae (Miyazaki *et al.*, 1998). Although the drinking rate is influenced by the temperature and the salinity of the surrounding medium (Tytler and Ireland, 1994), it has been shown that larvae continue to drink in low-salinity brackish water and even in water iso-osmotic to their body fluids (Tytler *et al.*, 1993; Guerreiro *et al.*, 2004).

Cod larvae treated with CCK-8s by daily immersions did not show elevated mortalities due to the daily handling when compared to untreated controls. Brown and Kim (1995) and Kim and Brown (2000) also successfully (in terms of improved survival and positive effects on gastrointestinal enzyme secretion) applied 1 hour-immersion treatment for the administration of thriiodothyronine and cortisol to larvae of Pacific threadfin, although treatment was only performed once. Strand and Dalmo (1997) studied the absorption of laminaran (an immunomodulator, a substance that is believed to alter the non-specific defence mechanisms in yolk-sac larvae), by immersion treatment that was applied on Atlantic halibut larvae for up to 10 days. Treatment by immersion has also been used in many studies dealing with the larval drinking activity of various fish species like *Aphanius dispar* (Skadhauge and Lotan, 1974), *Gadus morhua* (Mangor-Jensen and Adoff, 1987), *Oncorhynchus mykiss* (Tytler *et al.*, 1990), *Scophthalmus maximus* (Reitan *et al.*, 1998), *Morone saxatilis* (Grizzle and Cummins, 1999) and *Oreochromis mossambicus* (Lin *et al.*, 2000 and 2001).

Since this type of administration relies on the physiological drinking rate of fish larvae, results aught to represent real values under normal rearing conditions, without predominant stress influence. Immersion treatment was mentioned by Tytler *et al.* (1990) to offer a method for administering dietary supplements, or antigens for the purpose of

immunisation, and is an appropriate alternative for long-term nutritional experiments, easy to handle on a daily basis.

4.3　Methods for the combined analysis of CCK-8 concentration and tryptic activity

The methods performed for the analysis of CCK levels and tryptic activity were highly sensitive and allowed the use of small sample amounts, enabling the combination of both on a single newly hatched larva. This was essential in the present study, since the individual variability provides more detailed information about gut functionality and nutritional condition.

The preparation of the larval samples prior to their use in the respective analysis as well as the performance of both methods, are easy to handle. Attention must be paid to the step for CCK-8 extraction. Preliminary tests showed that even better results can be obtained from larvae homogenised in a dry-frozen (fluid nitrogen) condition, rather than in 50 µl of distilled water as practiced here. The supplement of water was preferably chosen because of time saving and reasons of precision, since dry homogenisation takes a lot more time and attention so as not to miss any unbroken larval tissue. Moreover, after adding the alcohol further homogenisation of missed tissue lumps is impossible. Yet, the amount of water used must be kept as small as possible, since the tests also showed that a dilution of the alcohol concentration used immediately after homogenisation, negatively influences the extraction success. Additionally, the higher the water content in the samples, the longer the duration of the drying step.

According to the manual of the CCK-RIA-kit, which was developed for plasma samples, the CCK-8 extraction is normally performed in ethanol. In this study with tissue samples methanol was used instead, since ethanol might decrease the activity of trypsin (Simon *et al.*, 1998). Another advantage of using methanol is that bigger CCK molecules (CCK 26-33), which can not be differentiated by the used RIA as described in the supplier's manual, are not as soluble as CCK-8 in this medium (Ichihara *et al.*, 1984).

In a previous study (Drossou *et al.*, 2006) a bioassay method based on the secretion effect of CCK on amylase from isolated rat acinar cells (Liddle *et al.*, 1984 and 1985; Kruse-Jarres *et al.*, 1989; Herzig *et al.*, 1997), was also successfully performed with fish larvae. In contrast to the CCK-RIA, the bioassay was more cost effective, but not less time consuming, and was complicated by the additional sacrifice of rats for the preparation of the acinar cells (Williams *et al.*, 1978; Höcker *et al.*, 1992). Additionally, the sensitivity of 1 pmol allowed only the measurement of pooled samples, so that a greater number of experimental individuals was needed, in order to get at least three statistically relevant

means. The lowest CCK concentration measured in the present study was 0.22 fmol/larva (corresponding to 0.44 pmol/l), which is within the sensitivity specification given by the supplier.

A major time limitation, restricting the number of processed larvae per day, was the use of the spectral-fluorometer *SFM 25* for the measurement of tryptic activity, which takes about 10 minutes to measure 4 samples. In the frame of another project during the same time, an opportunity was given to test and evaluate the sample measurement of tryptic activity on a 96-well-microtiter-filter-fluorometer *Fluoroscan Ascent®*, which allows the simultaneous measurement of 96 samples within a few seconds. This application enables a great deal of time saving during the performance of chemical analysis, as well as the use of even smaller sample amounts, and is now available for future studies.

4.4   Condition of larval batches used in the experiments, based on growth data

The size of larvae at the time of first feeding is shown in Table 11 for the species of Atlantic halibut, Atlantic herring and Baltic cod studied in the present work. All experimental groups of a single species showed comparable growth results. This was expected among the different groups of halibut and herring, since the treatment was of short-term, and larvae were micro-injected and sampled on the same day. Thus, a group differentiation in the growth data plots wouldn't have been necessary, but it enabled the proof of a randomly practiced sampling, and excluded the case of having used only small or only big larvae in one treatment group. A treatment effect on growth was also not evident in cod larvae that had been immersed in CCK-8s solutions over a period of 11 consecutive days.

Table 11: Mean standard length and dry weight (± S.D.) at or shortly after first feeding of larvae of the three species studied

Atlantic halibut 2 days after first feeding (40 dah)
12.65 ± 0.45 mm (n = 70)
0.58 ± 0.05 mg (n = 71)
Atlantic herring 3 days after first feeding (6 dah)
10.62 ± 0.55 mm (n = 76)
0.18 ± 0.06 mg (n = 76)
Baltic cod on the day of first feeding (3 dah)
4.37 ± 0.28 mm (n = 6)
0.26 ± 0.03 mg (n = 6)

The halibut larvae used in the present experiment were identified to have the "gaping jaw" disease, a deformity which has been shown to occur enhanced at temperatures higher

than 4°C (Pittman *et al.*, 1989 and 1990b; Lein and Poppe, 2005). "Gapers" are unable to close their mouth and to feed actively, so that mortality due to starvation follows after total absorption of the yolk-sac, which lasts longer than in healthy individuals due to decreased activity (Pittman *et al.*, 1990a). Although Morrison and MacDonald (1995) related pathogenic organisms present in the water and abrasion of the head by contact with the rearing tank wall to the development of "gaping jaws", the origin of this deformity remains unclear.

If feeding is initiated too late, larval energy resources will be used for maintenance and the digestive system will degenerate. Halibut larvae have a very long yolk-sac phase of 280-320°d (Gawlicka *et al.*, 2000) and thus, the time of first feeding, which was initiated here 38 days after hatching (250°d), has been subject of numerous studies. The day food was offered the first time by other researchers ranged mainly from 200 to 285°d (Næss *et al.*, 1995; Berg, 1997; Olsen *et al.*, 2000; Evjemo *et al.*, 2003; Rojas-García and Rønnestad, 2003; Solbakken and Pittman, 2004; Sæle *et al.*, 2003 and 2004). Lein and Holmefjord (1992) found that larvae first fed within the period of 200-265°d showed the highest feeding incidence. Harboe and Mangor-Jensen (1998) suggested the time between 260 and 290°d as being correct – in terms of aquaculture benefits - for initial feeding, and Gawlicka *et al.* (2000) based their conclusions on enzyme activity measurements indicating that halibut larvae should be first fed after 230°d but not later than 276°d. In the present study with "gaper" larvae, yolk reserves were visible during the entire experimental period (38-44 dah), but due to decreasing length and weight data from day 44 after hatching onwards, it can be concluded that only samples taken on 40 dah represent larvae with a sufficient nutritional supply. Thus, "gaper" halibut larvae unable to feed actively can be regarded as "normal" unfed yolk-sac individuals only until day 40 after hatching, although further morphological and/or physiological dysfunctions may be linked with this deformity (Morrison and MacDonald, 1995). An interesting approach would be to test whether food has a visible stimulation on growth and enzyme activities of these larvae, by keeping "gapers" in the presence and in the absence of food in the rearing tank.

At 6°C larvae of Atlantic herring hatched 21 days after fertilisation and were first fed 3 dah, when the mouth opening was observed. At 29 dah larval length and weight reached 19.05 ± 1.43 mm and 1.28 ± 0.40 mg (n = 86), respectively. Feeding on day 3 after hatching was also commenced by Folkvord *et al.* (2000). While the daily growth rate in standard length of about 0.4 mm/d recorded here, was also reported by Folkvord *et al.* (2000) for Norwegian Atlantic herring larvae, in that study larvae aged 25 dah had a dry weight of about 0.4 mg, which is less than half of the dry mass that was reached by larvae of the same age (0.94 ± 0.23 mg, n = 108) in the present experiment. Ueberschär (2000) recorded a standard length of about 13 mm from herring larvae aged 30 dah reared at temperatures between 13 and 17°C and fed with rotifers and *Artemia*. Thus, according to

the growth results the herring larvae used for micro-injection treatments had a good food supply and were in good nutritional condition.

Baltic cod larvae hatched 16 days after fertilisation and incubation at 7°C, a duration which is in accordance with results from Ueberschär (2000). The larvae were then kept at 6°C and were first fed at 3 dah, but feeding organisms were first observed in the larval guts at 5 dah. Although yolk-like rudiments were observed macroscopically until day 13 after hatching, the significant drop in dry weight at 6 dah in fed as well as in unfed larvae, probably marks the absorption of the nutritionally efficient part of the yolk, and is in agreement with Walden (2000) who mentioned that yolk-sac is used up at 6-8 dah. The fact that treated larvae showed the first weight loss one day earlier than untreated individuals, gives evidence for an enhanced yolk-sac absorption by exogenous administration of CCK-8s. Since the point of no return for cod larvae reared at 7°C is reported to be around 11 dah (Yin and Blaxter, 1986), the second weight loss on day 10 after hatching, which was also characterised by very low tryptic activities, suggests that the larvae had begun to starve, also explaining the stagnation in length increment. Considering though the significant differences in standard length and tryptic activity between fed and unfed individuals, it can be concluded that the larvae did feed on the organisms administered, but the food probably didn't meet the nutritional requirements of this species at the studied developmental stage, resulting in mass mortalities and the experimental termination at 15 dah. On the other hand, stagnating length from 8 to 13 dah was also recorded by Ueberschär (2000), and mass mortalities are mentioned in the literature between 12 and 16 dah, which level off later on (Yin and Blaxter, 1986; Olsen, 1997), so that at a higher availability of experimental individuals a longer observation of the cod larvae could have been possible.

4.5 Ontogenetic development of CCK content and tryptic activity in marine fish larvae

In order to give an overall impression of the species-specificity of CCK content and tryptic activity, the values of both were related to the larval dry mass at the time of first feeding, and are shown in Table 12 for untreated larvae of Atlantic halibut, Atlantic herring and Baltic cod studied in the present work.

Table 12: Mean individual (± S.D.) dry weight specific CCK content (in fmol mg^{-1}) and tryptic activity (in nmol min^{-1} mg^{-1}) at or shortly after first feeding of larvae of the three species studied

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Atlantic halibut 2 days after first feeding (40 dah)

19.53 ± 1.60 fmol mg^{-1} (n = 3)

2.92 ± 0.71 nmol min^{-1} mg^{-1} (n = 5)

Atlantic herring 3 days after first feeding (6 dah)

3.90 ± 0.23 fmol mg^{-1} (n = 3)

23.24 ± 17.80 nmol min^{-1} mg^{-1} (n = 5)

Baltic cod on the day of first feeding (3 dah)

2.91 ± 0.68 fmol mg^{-1} (n = 3)

0.46 ± 0.10 nmol min^{-1} mg^{-1} (n = 6)

</div>

CCK as well as tryptic activity were already detectable in embryonic stages of cod, at levels that reflected the starting depot in newly hatched larvae.

Unfed cod yolk-sac larvae showed an increase in tryptic activity until day 7 after hatching, demonstrating the existence of larval proteolytic digestive potential without enzymatic contribution from digested prey organisms.

The highest mean tryptic activities measured by Ueberschär (2000) within a period of 40 dah in herring larvae reared at 13-17°C on rotifers and *Artemia*, were about 3.5 nmol/min at 21 and 23 dah. Herring larvae reared on natural zooplankton in the present study exceeded 7 nmol/min from day 20 after hatching onwards, proving that tryptic activity reflects the food quality.

Considering the tryptic activities of cod larvae, individuals in the present study showed a much slower development than described by Ueberschär (2000), although culture conditions were almost the same, with exception of 2°C difference in the rearing temperature. In that batch, individuals reached a tryptic activity of about 1 nmol/min at 4 dah, which fell then until 9-10 dah and started increasing significantly thereafter. In comparison, cod larvae in the present study showed lower tryptic activities and the described development was shifted 3-4 days forward.

In larvae of Atlantic herring and Baltic cod a feedback mechanism between CCK content and tryptic enzyme activity was evident. The demonstration of this interaction was only possible due to the daily larval sampling and data plot. In contrast, when considering

different sampling times within one day (diel variability) the CCK content and the tryptic activity in herring larvae showed quite similar trends rather than an exact opposite pattern, and this is in agreement with the results obtained by Koven *et al.* (2002). In the present study, the daily oscillation range was shown to depend on the ontogenetic development, and was increasing with age for both parameters.

While starvation resulted to the decline of the CCK content and the tryptic activity in all three larval species, so that both parameters can be considered as short-term indicators for changes in the food supply, in regularly fed cod larvae trypsin seemed to be the main trigger for CCK production and/or release, rather than the intake of food.

In an attempt to combine the above observations, the following action pattern including short- and long-term oscillations can be concluded:
- Short-term development (diel variability): as a consequence of food intake CCK release stimulates the secretion of pancreatic enzymes. After completion of the digestion process concentrations fall. The secretion levels of CCK and Trypsin depend on the developmental stage, since older larvae need higher concentrations (individual concentrations are meant here and not weight-specific concentrations).
- Long-term development (the same sampling time over several days): CCK in regularly fed larvae increases when tryptic activity reaches starvation levels – which change according to the developmental stage -, and remains on a base level when trypsin secretion is sufficient to facilitate digestion. Under starvation conditions though, this regulatory mechanism is being suppressed, probably in order to minimise the energetic loss.

4.6 Effects of external CCK-8s and PHA on larval levels of CCK and tryptic activity

In contrast to tryptic activity, the CCK content in herring was higher in larvae injected with physiological saline than in untreated individuals, suggesting that the distension of the gut wall was not a trigger for CCK secretion. Koven *et al.* (2002) micro-injected Atlantic herring larvae aged 4-11 dah with physiological saline and came to the same conclusion, which they also assumed for trypsin secretion. In contrast, tryptic activity was affected by the injected volume in the present study, indicating that triggering of CCK and trypsin release is not necessarily combined in fish larvae, or another mechanism is involved. In mammals, the presence of a CCK-releasing factor was hypothesised and led to the discovery of the luminal CCK-releasing factor (Spannagel *et al.*, 1996) and diazepam-binding inhibitor (Herzig *et al.*, 1996), as two potential peptides that may regulate CCK release and mediate pancreatic enzyme secretion: under basal conditions where small amounts of trypsin are present, the CCK-releasing factor is degraded and CCK secretion is low; ingested food,

particularly protein, which is a substrate for trypsin, temporarily binds trypsin and prevents degradation of the CCK-releasing factor; intact CCK-releasing factor in turn stimulates the release of CCK, which enhances pancreatic enzyme secretion (Liddle, 1997 and 2000; Herzig, 1998). However, the mechanisms regulating the production and secretion of CCK-releasing factors are largely unknown. The existence of a CCK-releasing factor was also supported in studies about lipid and protein digestion in seabass larvae (Zambonino-Infante and Cahu, 1999; Cahu *et al.*, 2004).

The administration of exogenous CCK-8s resulted in increasing levels of larval CCK and tryptic activity in individuals of halibut aged 40 dah and of cod aged 6-11 dah. It is not obvious, why cod larvae were not affected prior to 6 dah, although treatment began at 4 dah. A minimum treatment duration of 48 hours can't be concluded as necessary, since halibut larvae reacted on the same day they were micro-injected. Also, long-term treated cod larvae showed elevated CCK concentrations and tryptic activities only after the immersions and not before, suggesting that a single treatment can manipulate physiological levels of the two parameters. Drossou *et al.* (2006) treated Nile tilapia larvae with PHA on a long-term scale and achieved effects on CCK content and tryptic activity not before 18 dah. Indicatively, the ontogenetic development is the predominant factor influencing the larval digestive system during the very first days after hatching, and the absorption rate of an externally applied substance depends on the developmental stage. Thus, studies with different concentration series have to be performed in order to determine dose ranges that can influence humoral and enzymatic levels immediately after hatching. Einarsson *et al.* (1997) achieved increasing tryptic activities for up to 25 minutes, due to treatments with exogenous CCK in *in vitro* experiments with post-smolt Atlantic salmon. Thus, in future tube-feeding studies with CCK-8s, sampling times of less than 2 hours after treatment should be tested additionally, especially in newly hatched larvae.

Although for cod larvae the concentration of the CCK-8s treatment solution was the same every day, the measured CCK concentrations varied daily, and this strengthens the assumption that the amount of hormone that can be absorbed depends on the developmental stage and most commonly also on the nutritional condition of a larva.

In cod larvae effects on CCK content and tryptic activity were obtained in the group treated with the lower but not with the higher CCK-8s concentration, indicating an overdose of CCK-8s in the second group. It is not surprisingly that the effects of externally applied CCK-8s can be dose dependent. In the case of unaffected tryptic activity, it can be assumed that a CCK-8s overdose can act as a laxative and cause postprandial excretion without further utilisation of the hormone. It is difficult to explain though, why the CCK levels were also not elevated. Even if there was too much hormone in the solution, one

would expect that some ingestion and absorption must have taken place through drinking, and that absorption would follow a saturation kinetics model similarly to the proposal of Barr *et al.* (2001) and Applebaum and Rønnestad (2004) for protein and free amino acid absorption. Morais *et al.* (2005) mentioned for herring larvae tube-fed with lipids that there is a limit to the lipid inclusion level, above which increased evacuation without absorption was observed. However, in that study it is not possible to differentiate whether the results of the authors were attributed to a higher concentration or a higher volume.

Larvae of herring treated with CCK-8s showed the highest CCK concentrations already 2 hours after micro-injection, indicating that absorption of the externally applied hormone had been completed by that time and utilisation followed thereafter. Apart from this observation and although single differences were recorded, herring larvae didn't show clear effects due to the treatments with CCK-8s.

In general, the daily oscillation variability made it difficult to distinguish whether elevated CCK concentrations or tryptic activities were attributed to the normal daily rhythm or to an applied treatment. Additionally, since herring larvae were analysed without the head – in order to exclude brain CCK concentrations – the measured CCK values may represent a quite small range that complicated the recording of statistical differences: according to Rojas-García *et al.* (2006) CCK levels in the head compartment represented more than 80%, in the gut only about 10% of the whole body CCK concentration in herring larvae. In other tube-feeding studies, micro-injections were performed only on herring larvae previously starved for 24 hours (Morais *et al.*, 2005), in order to evacuate the digestive system. Herring larvae used in the present study were treated immediately after removal from the rearing tank, so that the digestive process of regularly feeding individuals complicated the interpretation of the results. On the other hand, conclusions about absorption and utilisation in starving fish larvae are not appropriate to identify normal physiological reactions. A certain hormonal dose might cause an effect in starving larvae but not in individuals with normal endogenous levels of the same hormone.

Another possibility for not obtaining obvious treatment differences in herring larvae could have been the injection volume used. Tytler *et al.* (1993) determined a drinking rate of 9.2 nl per hour in 11 days old Baltic herring at 10°C in full strength seawater (salinity of about 32). It could be assumed that this volume, which corresponds to the volume micro-injected in herring larvae within a few seconds, might have been too high for the larvae to deal with at once, and that enhanced peristaltic movements – which were observed as a consequence of the injection – resulted to unabsorbed excretion. On the other hand, the micro-injected volume of 9.2 nl corresponds to one *Artemia* nauplius (Rønnestad *et al.*, 2000b). Since more than one prey organisms have been counted in the larval gut of

feeding herring, and Rønnestad *et al.* (1998) quantified up to 27 *Artemia* in the gut of first fed halibut larvae, it seems unlikely that the size of the injected droplet was to big.

The administration of PHA achieved elevated CCK concentrations in halibut and herring larvae 6-8 hours after treatment, but failed to affect the larval tryptic activity within the incubation periods applied. A delayed reaction was expected, since the PHA treatment represents an indirect stimulation of CCK release. Considering the results obtained by Rådberg *et al.* (2001), who suggested gut maturation and pancreatic growth due to PHA intake in piglets, it is most likely that only a repeated administration of PHA can result in effects on the pancreatic enzyme secretion. However, attention must be paid in keeping dosages low, since anti-nutritional effects have been associated with the administration of PHA, like changes in the intestinal microbial flora, morphological damage of the villi, malabsorption and poor growth (Banwell *et al.*, 1984 and 1985; Calrvalho and Sgarbieri. 1998; Francis *et al.*, 2001). These harmful effects can be removed by prolonged cooking (Muzquiz *et al.*, 1999) and seem to be dose-dependent (Otte *et al.*, 2001). Drossou *et al.* (2006) could prove that a daily PHA dose lower than 0.05% of the wet body weight did not have an impact on the growth of long-term treated Nile tilapia larvae.

4.7   Conclusions

The micro-tube feeding administration type was evaluated as a proper method to simulate single meal events in fish larvae. The survival rate of the treated individuals depends on the developmental stage and on morphological characteristics of the mouth and the gut. For long-term observations on a greater number of experimental individuals, treatment through repeated immersion should given preference.

The combined preparation of samples for their use in a radioimmunoassay for CCK quantification and in a fluorometric assay for measurement of tryptic activities was possible, and enables to obtain information about two parameters that play a key role in the larval digestive physiology, from individual fish larvae.

In the present study, a feedback mechanism between CCK and tryptic activity was demonstrated for the first time in marine fish larvae. According to the obtained results from larvae of halibut, herring and cod, a short and a long-term action pattern was described for CCK and tryptic activity. Additionally, it can be suggested that the presence or absence of a sufficient food quantity determines whether the feedback regulation between CCK and trypsin secretion – within which the level of trypsin activity represents the long-term trigger for the amount of CCK release – is active or not. The results also support the existence of a CCK-releasing factor postulated by other researchers.

The administration of CCK-8s affected larval CCK and tryptic activity levels on a short-term scale, but did not result in elevated endogenous levels over a longer period. Thus a single treatment with CCK-8s is sufficient to influence the CCK content and the tryptic activity of a fish larva. Total absorption of externally applied CCK-8s takes up to 4 hours after treatment, but the rate of absorption depends on the developmental stage and the nutritional condition of the larvae. There was also evidence for an overdose of an administered CCK-8s concentration on cod larvae, suggesting that the effects of this hormone are dose-dependent.

Although a stimulating effect of PHA on the CCK content was observed after a single micro-injection, it is possible that only a repeated treatment with this plant protein can achieve a stimulation of the tryptic activity, probably indirectly through stimulation of pancreatic growth.

In all three species the ontogenetic development was found to have the predominant effect on the trends of CCK content and tryptic activity. The different treatments applied, affected the values of CCK and tryptic activity but the developmental trends remained.

Summary

The present study focused on the interactions between the gastrointestinal hormone cholecystokinin (CCK) and the activity of the pancreatic enzyme trypsin, in larval stages of marine fish species from hatching onwards. A feedback relationship has been proven between these two parameters in mammals and adult fishes, but not yet in larval fish stages.

In order to test whether it is possible to manipulate the endogenous CCK level and consequently the tryptic enzyme activity in the gut of marine fish larvae, a direct way through treatment with the physiological hormone CCK-8s and an indirect way through administration of the plant protein phytohemagglutinin (PHA) as a CCK releasing stimulus were chosen. Additionally, two different applications were performed. Larvae of Atlantic halibut (*Hippoglossus hippoglossus*) and Atlantic herring (*Clupea harengus*) were short-term treated with single *in vivo* micro-injections (micro-tube feeding method) allowing the direct administration of the test solutions into the larval gut, and were then sampled after different incubation periods. Totally untreated larvae and individuals micro-injected only with physiological saline were sampled as controls. A long-term treatment was performed on larvae of Baltic cod (*Gadus morhua*), which were immersed daily for 1½ hour in CCK-8s solutions of two different concentrations (PHA was not tested here) over a period of 11 days. The treated cod larvae were compared with totally untreated individuals as a control. Data of standard length, dry weight, CCK content and tryptic enzyme activity were obtained from each sampled individual. For the chemical analysis of CCK level (by radioimmunoassay) and tryptic activity (by a fluorometric method) on individual larvae, a combined preparation protocol was tested, allowing the use of small sample amounts.

The micro-tube feeding administration type was evaluated as an appropriate method to simulate single meal events in fish larvae. The survival rate of the treated individuals depends on the developmental stage and on morphological characteristics of the mouth and the gut. For long-term observations on a greater number of experimental individuals, treatment through repeated immersion should given preference.

The combined preparation of samples for their use in a radioimmunoassay for CCK quantification and in a fluorometric assay for measurement of tryptic activities is possible, and enables to obtain information about two parameters that play a key role in the larval digestive physiology, from individual fish larvae.

A feedback mechanism between CCK and tryptic activity was demonstrated for the first time in marine fish larvae. According to the obtained results from larvae of halibut, herring

and cod, the following short and a long-term action pattern was postulated for CCK and tryptic activity:

- Short-term development (diel variability): as a consequence of food intake CCK release stimulates the secretion of pancreatic enzymes. After completion of the digestion process concentrations decrease. The secretion levels of CCK and trypsin depend on the developmental stage.
- Long-term development: the CCK secretion levels in regularly fed larvae increase when tryptic activity reaches starvation levels, and remains on a base level when trypsin secretion is sufficient to facilitate digestion.

Additionally, it was suggested that the presence or absence of a sufficient food quantity determines whether the feedback regulation between CCK and trypsin secretion remains functional or not. The results also supported the existence of a CCK-releasing factor postulated by other researchers.

The administration of CCK-8s affected larval CCK and tryptic activity levels on a short-term scale and did not result in elevated endogenous levels over a longer period, thus a single treatment with CCK-8s is sufficient to influence the CCK content and the tryptic activity of a fish larva. Total absorption of externally applied CCK-8s takes up to 4 hours after treatment, but the rate of absorption depends on the developmental stage and the nutritional condition of the larvae. There was also evidence for an overdose of an administered CCK-8s concentration, suggesting that the effects of this hormone are dose-dependent.

Although a stimulating effect of PHA on the CCK content was observed after a single micro-injection, it is suggested that only a repeated treatment with this plant protein can achieve a stimulation of the tryptic activity.

In all three species the ontogenetic development was found to have the predominant effect on the trends of CCK content and tryptic activity. The different treatments applied, affected the values of CCK and tryptic activity but the ontogenetic developmental trends remained visible.

Zusammenfassung

In der vorliegenden Arbeit wurden die Interaktionen zwischen dem gastrointestinalen Hormon Cholecystokinin und der Aktivität des pankreatischen Enzyms Trypsin in larvalen Stadien mariner Fischarten untersucht, vom Zeitpunkt des Schlupfs an. Zwischen diesen beiden Parametern wurde ein Feedback Mechanismus in Säugetieren und adulten Fischen gezeigt, der jedoch in Fischlarven bisher nicht bekannt war.

Die Möglichkeit der Manipulation endogener CCK Konzentrationen und folglich der Trypsinaktivität im Darm mariner Fischlarven wurde direkt durch die Applikation des physiologischen Hormons CCK-8s und indirekt durch die Verabreichung des pflanzlichen Proteins phytohemagglutinin (PHA), dass als Stimulans für die CCK Ausschüttung bekannt ist, getestet. Die Behandlung erfolgte durch zwei verschiedene Verabreichungsmethoden. Larven von Heilbutt (*Hippoglossus hippoglossus*) und Hering (*Clupea harengus*) erfuhren einmalige Behandlungen durch *in vivo* Mikroinjektionen („micro-tube feeding" Methode), welche die direkte Verabreichung der Testlösungen in den larvalen Darm ermöglichten, und wurden nach verschiedenen Inkubationszeiten beprobt. Als Kontrollgruppen dienten völlig unbehandelte Larven und solche, die nur mit physiologischer Kochsalzlösung injiziert wurden. Eine längere Behandlung wurde an Dorschlarven (*Gadus morhua*) angewandt, welche täglich in CCK-8s Lösungen zweier verschiedener Konzentrationen für 1½ Stunden gebadet wurden und zwar über einen Zeitraum von 11 Tagen. Die behandelten Larven wurden dann mit völlig unbehandelten Individuen verglichen.

Daten von Standardlänge, Trockengewicht, CCK Konzentration und Trypsinaktivität wurden von jeder einzelnen Fischlarve erhoben. Für die chemische Analyse zur Quantifizierung der individuellen CCK Konzentration (mittels eines Radioimmunoassays) und Trypsinaktivität (mittels einer fluorometrischen Methode) wurde ein Protokoll getestet, dass die kombinierte Bearbeitung und die Anwendung kleinster Mengen von Proben ermöglichte.

Der „micro-tube feeding" Applikationstyp wurde als eine geeignete Methode zur Simulation von Einzelfütterungsereignissen in Fischlarven evaluiert. Die Überlebensrate der mikroinjizierten Larven hängt vom Entwicklungsstadium sowie von morphologischen Charakteristika des Mauls und des Darms ab. Für Langzeituntersuchungen an einer größeren Anzahl experimenteller Individuen dagegen, eignet sich die Behandlung durch wiederholtes Baden in einer Lösung mit dem gewünschten Verabreichungsstoff besser.

Die kombinierte Vorbereitung der Proben zur Anwendung sowohl im CCK-Radioimmunoassay als auch in der Messmethode der Trypsinaktivität, ist leicht zu

handhaben und ermöglicht die Erfassung beider Parameter, die eine Schlüsselrolle in der larvalen Verdauungsphysiologie spielen, an einer und der selben Larve.

Zwischen der Entwicklung der CCK Konzentration und der Trypsinaktivität konnte ein Feedback Mechanismus an marinen Fischlarven erstmalig gezeigt werden. Anhand der Ergebnisse von Heilbutt-, Herings- und Dorschlarven konnten ein Kurzzeit- und ein Langzeit-Muster der Interaktionen zwischen CCK Konzentration und Trypsinaktivität beschrieben werden:

- Kurzzeitentwicklung: die CCK Ausschüttung, welche durch die Futteraufnahme angeregt wird, stimuliert die Sekretion von Verdauungsenzymen aus dem Pankreas. Die Konzentrationen fallen nach Beendigung des Verdauungsprozesses. Die Höhe der freigesetzten CCK- und Trypsinmengen hängt vom Entwicklungsstadium ab.
- Langzeitentwicklung: die Menge des freigesetzten CCK in regelmäßig gefütterten Fischlarven nimmt zu, wenn die Trypsinaktivität auf dem Niveau hungernder Individuen liegt, und bleibt auf einem niedrigen Basisniveau sobald die Sekretion von Trypsin ausreichend hoch ist, um die Verdauung zu gewährleisten.

Zusätzlich konnte gezeigt werden, dass die Aufrechterhaltung des Interaktionsmechanismus zwischen CCK und Trypsin von der Menge des Futters abhängig ist, das zur Verfügung steht. Die Ergebnisse aus dieser Arbeit unterstützen außerdem die Aussage anderer Wissenschaftler über die Existenz eines „CCK-releasing" Faktors.

Die Verabreichung von CCK-8s hatte einen Einfluss auf die larvale CCK Konzentration und die Trypsinaktivität, der jedoch nicht anhaltend über einen längeren Zeitraum war, d.h. eine einzelne Hormonbehandlung reicht aus, um endogene Konzentrationen von CCK und Trypsinaktivität zu manipulieren. Die Absorption von extern appliziertem CCK-8s dauert bis zu 4 Stunden nach der Behandlung, und die Absorptionsrate hängt vom Entwicklungsstadium und dem ernährungsphysiologischen Zustand der Larve ab.

Ein stimulierender Effekt auf die CCK Konzentration wurde auch durch einzelne Mikroinjektionen mit PHA erreicht, doch möglicherweise bedarf es wiederholter Behandlungen mit diesem Protein, um auch eine erhöhte Trypsinsekretion zu erzielen.

Die Entwicklung der CCK Konzentration und der Trypsinaktivität wurde in allen drei Larvenarten hauptsächlich ontogenetisch beeinflusst. Die verschiedenen Behandlungen haben absolute Werte beeinflusst, doch die ontogenetischen Entwicklungstrends blieben erkennbar.

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