Effect of free amino acids and natural extracts on the tryptic enzyme activity of European sea bass larvae (*Dicentrarchus labrax* L.)

Master thesis

presented by Inken Hanke

University of Hamburg

Institute for Hydrobiology and Fisheries Science

1. Thesis adviser: Prof. PhD. Myron Peck

2. Thesis adviser: Dr. rer. nat. Bernd Ueberschär

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Summary

Fish larvae of most marine species common in aquaculture lack a functional stomach at onset of first feeding and the entire digestive tract go through considerable developmental changes until metamorphosis. In this period larval digestion takes place in the gut, in which trypsin functions as a key proteolytic enzyme. Considering the limited digestive capacity during these developmental changes it becomes clear that adequate feeding during the entire larval development is still challenging. In the last decades first promising achievements can be reported to optimize larval rearing by weaning larvae from live food and feeding microparticulate diets (Micro Diets, MD) instead. However, despite these achievements further investigations to optimize MD in terms of attractiveness and particularly digestibility are necessary, because for many marine species the complete substitution of live food is still not recommended.

This study was designed to identify trigger substances, which have the potential to stimulate the trypsinogen secretion and consequently facilitating an increasing tryptic enzyme activity in the larval gut. Four different trigger solutions were tested, two amino acid mixtures: betaine – alanine – arginine – glycine (BAAG) and glycine – betaine (GB); and two natural extracts: *Artemia* rearing water (ARW) and an extract of *Mytilus edulis* (MY). European sea bass larvae (*Dicentrarchus labrax*) were exposed to these trigger solutions for 30 minutes. Samples were taken after 10, 20 and 30 minutes at the age of 14 / 15 days post hatch (dph), 21 / 22 dph and 27 / 28 dph.

In order to acknowledge if a circadian rhythm of the tryptic enzyme activity can have an impact on the evaluation of the trigger experiments, the diel rhythm in tryptic enzyme activity of 13 dph, 20 dph and 27 dph old sea bass larvae was examined. Diel rhythm experiments revealed feeding induced diel variations in tryptic enzyme activity of fed sea bass larvae. Unfed larvae (one day deprived of food) maintain a low and steady basic level in tryptic enzyme activity during the day compared to fed larvae.

The trigger experiment revealed higher tryptic enzyme activities for sea bass larvae, which were exposed for 10 – 30 min to BAAG and for 10 - 20 min to ARW at 28 dph. No clear response in tryptic enzyme activity was observed for larvae, which were exposed to GB and MY. In general, in younger larvae no (clear) response was observed. It was supposed that tested concentration of GB and MY might be too low to trigger the trypsinogen secretion, at least for older larvae. It was further assumed, that for the early larval stages an additional

trigger such as mechanical stimuli inside of the gut due to food ingestion might be necessary to trigger the secretion of trypsinogen.

The results of the study can be useful to improve the digestibility, palatability and attractiveness of microdiets by supplementation of positively tested trigger substances such as BAAG. In future studies, emphasis should be put on the question, which concentrations of suggested trigger substances are most effective.

Zusammenfassung

Marine Larven von in Aquakulturen gehälterten Fischarten haben zu Beginn der ersten Futteraufnahme noch keinen funktionalen Magen und der gesamte Verdauungstrakt durchläuft beträchtliche entwicklungsbedingte Änderungen bis zur Metamorphose. In dieser Zeit findet die Verdauung im Larvendarm statt, wobei Trypsin als eines der wichtigsten proteolytischen Enzyme fungiert. Angesichts der begrenzten Verdauungsleistung während dieser entwicklungsbedingten Änderungen wird deutlich, dass angemessene Ernährung / Fütterung während der gesamten Larvenentwicklung eine große Herausforderung darstellt. In den letzten Jahrzehnten konnten erste vielversprechende Erfolge verzeichnet werden, die Larvenzucht zu optimieren, durch das Absetzten von Lebendfutter und stattdessen die Fütterung von Microdiets (MD). Trotz dieser Erfolge sind jedoch weiter Untersuchungen notwendig, Microdiets hinsichtlich ihrer Attraktivität und Verdaulichkeit zu optimieren, da für viele marine Arten der komplette Ersatz von Lebendfutter mit MD noch nicht empfehlenswert ist.

Die vorliegende Studie wurde durchgeführt, um Substanzen zu identifizieren, die die Trypsinogensezernierung stimulieren können und somit einen Anstieg der Trypsinaktivität im Larvendarm ermöglichen. Es wurden vier verschiedene Trigger - Substanzen getestet, zwei Aminosäuremischungen: Betain – Alanin – Arginin – Glycin (BAAG) und Glycin – Betain (GB); und zwei Extrakte: Artemien-Aufzuchtswasser (ARW) und ein Extrakt aus Miesmuscheln (MY). Dafür wurden Wolfsbarschlarven (*Dicentrarchus labrax*) im Alter von 14 / 15, 21 / 22 und 27 / 28 Tagen diesen Trigger - Substanzen für 30 Minuten ausgesetzt und es wurde nach 10, 20 und 30 Minuten Proben entnommen.

Um zu überprüfen, ob ein Tagesrhythmus der Trypsinaktivität einen Einfluss auf die Auswertung der Trigger-Experimente haben könnte, wurde der Tagesrhythmus der Trypsinaktivität von 13, 20 und 27 Tage alten Wolfsbarschlarven untersucht. Die Tagesrhythmus - Experimente zeigten einen durch Fütterung ausgelösten Tagesrhythmus in der Trypsinaktivtät der Wolfsbarschlarven auf. Nicht gefütterte Wolfsbarschlarven (für einen Tag) zeigten ein niedriges und konstantes Basis - Trypsinlevel über den Tag, verglichen mit gefütterten Larven.

Während der Trigger – Experimente konnte eine erhöhte Trypsinaktivität bei 28 Tage alte Wolfsbarschlarven, die für 10 – 30 Minuten BAAG oder für 10 – 20 Minuten ARW ausgesetzt waren, beobachtet werden. Keine eindeutige Reaktion in der Trypsinaktivität wurde bei Larven, die GB oder MY ausgesetzt waren, festgestellt. Bei jüngeren Larven wurde im Allgemeinen keine Reaktion auf die Trigger festgestellt. Es wird vermutet, dass die getestete Konzentration von GB und MY eventuell zu niedrig gewesen sein könnten, um die Trypsinsezernierung zu stimulieren, zumindest für ältere Larven. Jüngere Larvenstadien benötigen möglicherweise einen zusätzlichen Trigger, wie zum Beispiel einen mechanischen Reiz durch Nahrungsaufnahme, um die Trypsinsezernierung zu stimulieren.

Die Erkenntnisse dieser Studie können genutzt werden, um die Verdaubarkeit und Attraktivität von Microdiets zu verbessern, durch die Ergänzung von positiv getesteten Trigger - Substanzen wie BAAG. In zukünftigen Studien sollte der Schwerpunkt auf die Frage gelegt werden, welche Konzentrationen der genannten Substanzen am effektivsten sind.

List of abbreviations

ANOVA	Analysis of variance
ARW	*Artemia* rearing water
BAAG	mixture of betaine, alanine, arginine and glycine
BSA	bovine serum albumin
BZ-L-Arg-MCA	Na-benzoyl-L-arginine-4-methyl-coumarinyl-7-amide
CCK	cholecystokinin
DHA	docosahexaenoic acid
DMSO	dimethylsulfoxide
dph	day post hatch
EPA	eicosapentaenoic acid
FAA	free amino acids
GB	mixture of glycine and betaine
GMA	Gesellschaft für Marine Aquakultur
h	hour
L	litre
MD	microdiets
min	minutes
MY	extract of *Mytilus edulis*
NTU	nephelometric turbidity unit
PSU	practical salinity units
SD	standard deviation
µm	micrometer
°C	Degrees Celsius

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1 Introduction

1.1 European aquaculture food fish production

Aquaculture food fish production in Europe has stagnated its growth since the early 2000s, particularly compared with the substantial increase of the global aquaculture production. Increasing production in Europe is only reported for salmon in Norway in the last decade. In 2012, Europe produced 4.32 % (in volume) of the world aquaculture (food fish) production and of that the 28 members of the European Union (EU) produced only 1.89 % (FAO, 2014). However, EU aquaculture fish production mainly focuses on high-value marine fish species in contrast to the high production of lower-value species (e.g. cyprinidae) in many Asian countries. Dominating species in the EU production are salmonids and coastal species such as gilthead sea bream (*Sparus aurata*) and European sea bass (*Dicentrarchus labrax*) (STECF, 2013).

Key target for an efficient and competitive expansion of the European aquaculture industry is an enhanced production of high quality and healthy fry (Valente et al., 2013). Although adequate quantities of fish larvae are produced, for many marine species larval growth rates are still below their potential and survival of larvae is not consistently high (Valente et al., 2013). For instance several Mediterranean hatcheries have survival rates of around 10 % (Valente et al., 2013). Such reports indicate that there are still significant gaps in our knowledge concerning optimal nutritional and culture conditions in larval stages.

1.2 Live food vs. Microdiets

Fish larvae undergo dramatic morphological and physiological changes during ontogenetic development. Therefore, an adequate nutritional supply is essential for larvae to survive, develop and grow properly (Hamre et al., 2013). Larval diet must offer a balanced composition of various categories of nutrients. For instance larval diet should offer an adequate supply of free amino acids (FAA), because FAA have an important role during larval development in terms of body protein synthesis and as a source of energy (Rønnestad et al., 1999). Another important nutritional component for larval diets are essential fatty acids. It is known that deficiencies in fatty acids negatively affect larval growth, survival and activity (Izquierdo, 1996).

One approach to improve larval rearing techniques, particularly to fulfill nutritional demands, concerns the dependence of many marine species on live food during early life stages. Rearing phytoplankton and zooplankton as live food for fish larvae generates various problems, for instance the varying nutritional quality of live food. *Artemia* nauplii have for instance very limited and variable amounts of eicosapentaenoic acid (EPA) and no docosahexaenoic acid (DHA) and must therefore be enriched with these fatty acids. Enrichments are commonly used to increase the nutritional value of live food. However, in cases such as *Artemia* nauplii it is difficult to reach high levels of DHA even with enrichments, due to the natural tendency of *Artemia* to retro-convert DHA into EPA (Hamre et al., 2013). Additionally, the use of enrichments on a commercial scale lead to high production costs (Conceição et al., 2010).

An alternative for live food are microparticulate diets (Micro Diets, MD) with constant quality and balanced formulations to fulfill larval requirements (nutrition etc). During the last decades elaborated research on developing proper MD for larval rearing was performed. Several studies showed the possibility to replace live food with MD partially (co-feeding) to rear marine fish larvae (Baskerville-Bridges and Kling, 2000; Ben Khemis et al., 2003; Engrola et al., 2009; Kanazawa et al., 1989; Kolkovski et al., 1997b; Pedro Cañavate and Fernández-Díaz, 1999; Rosenlund et al., 1997). Currently, first promising progress is reported by feeding gilthead sea bream larvae with MD exclusively (pers. comm., B. Ueberschär). Additionally, even first commercial-scale trials in different hatcheries demonstrated equal or enhanced survival / growth rates of sea bream larvae fed with MD exclusively, compared to larvae fed with *Artemia*.[1] Despite these promising achievements further investigations to optimize MD are necessary, because for many marine species the complete substitution of live food, particularly for the first days of feeding, is still not recommended (Conceição et al., 2010; Hamre et al., 2013).

There are several reasons why MD currently cannot be applied for many marine species from onset of exogenous feeding without co-feeding live prey such as rotifers or *Artemia*. There are still gaps in knowledge about nutritional requirements (lipids, fatty acids, etc.), feeding behavior (detection, capture, etc.) and digestive physiology of the early life stages of marine fish larvae, although various studies already covered these topics.

[1] ©Skretting a Nutreco company
http://www.skretting.co.uk/internet/SkrettingUKIreland/webInternet.nsf/wPrId/67FCB930028304A9802576650056CA11!OpenDocument [cited 17 March 2015]

1.3 Digestive physiology of marine fish larvae

For marine fish larvae commonly grown in aquaculture, the digestive tract is still developing at onset of exogenous feeding. These types of larvae, defined as altricial, develop a functional stomach at metamorphosis, the transition period when larvae become juveniles (Govoni et al., 1986; Rønnestad et al., 2013). Several studies have previously described the development of the digestive tract and recent studies reconstructed and confirmed these perceptions with new techniques like 3D models (Dabrowski, 1984; Govoni et al., 1986; Kamisaka and Rønnestad, 2011).

In the following the generalized development of the digestive tract of altricial larvae is described. At hatching, the digestive tract of the larva is a straight, closed and undifferentiated tube (Dabrowski, 1984; Govoni et al., 1986). The mouth and anus are undeveloped and will be formed later during the yolk-sac phase (Kjorsvik et al., 2004). By the end of yolk sac absorption, the gut will extend and differentiate into various sections the buccopharynx, foregut, midgut and hindgut (Govoni et al., 1986; Kjorsvik et al., 2004) (Figure 1). Generally, the gut coils into a loop before exogenous feeding starts (Rønnestad et al., 2013). Accessory digestive organs such as liver and pancreas are functional at the end of yolk absorption. Until onset of metamorphosis the digestive tract elongates and folding of the mucous membrane takes place to increase the absorptive capacity (Kjorsvik et al., 2004; Rønnestad et al., 2013). The last major morphological change of the digestive tract constitutes the development of a stomach and pyloric cacea from the posterior foregut and can be considered as the end of the transition from larva to juvenile (Govoni et al., 1986; Rønnestad et al., 2013). Variations in time and characteristics of the described developmental phases occur among different species.

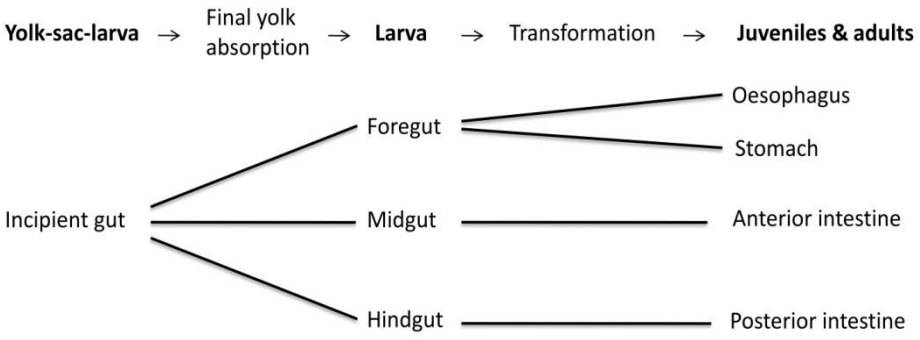


Figure 1: Chronological order of differentiation of the digestive tract (reprinted from Govoni et al., 1986) for altricial larvae.

With absence of a functional stomach the digestion at onset of exogenous feeding of altricial larvae takes place in the gut. Thereby digestive enzymes from the exocrine part of the pancreas like trypsin, chymotrypsin, lipase and amylase are important for early life stages to

break down macromolecular food particles (Kjorsvik et al., 2004). Trypsin is considered to be a key proteolytic enzyme in this period (Rønnestad et al., 2013; Zambonino Infante and Cahu, 2001). Trypsin is an endoproteinase and hydrolyses proteins by breaking peptide bonds of non-terminal amino acids, mainly after the position of basic amino acids (e.g. lysine, arginine). Moreover, trypsin activates its own precursor and the precursors of other pancreatic enzymes in the gut lumen by cleaving peptide bonds (Light and Janska, 1989; Zambonino Infante and Cahu, 2001). Trypsin is produced in the pancreas in an inactive precursor, called trypsinogen. The exocrine pancreas secretes trypsinogen into the gut lumen. Although many details about the regulation of larval digestion are still unknown, several studies in the last decade suggested that the pancreatic secretion is regulated by the peptide hormone cholecystokinin (CCK) (Rønnestad et al., 2013). CKK is produced in endocrine cells of the gut. It is suggested that the release of CKK is stimulated either mechanically by the ingestion of food or through a chemical stimuli (Koven et al., 2002; Rønnestad et al., 2013; Webb and Holt, n.d.). CCK is released into the circulatory system, targeting cells in the pancreas and thus stimulating the release of secretions (i.a. trypsinogen) into the gut lumen. In contrast trypsin acts as a feedback control for the CCK release of the endocrine cells (Rønnestad, 2002; Tillner et al., 2013).

The level of the tryptic enzyme capacity differs among marine species but increases with increasing larval size (Rønnestad et al., 2013; Ueberschär, 2006). Tryptic enzyme activity can already be detected before onset of exogenous feeding, at hatching or between 2 – 4 dph (Lazo et al., 2000; Ribeiro et al., 1999; Zambonino Infante and Cahu, 1994). Tryptic enzyme activity does not increase linear during ontogentic development. Fluctuations in tryptic enzyme activity were observed during the first days/weeks after hatching for different species (Ribeiro et al., 1999; Suzer et al., 2006; Ueberschär, 1995; Ueberschär, 1993; Zambonino Infante and Cahu, 1994). Ueberschär (2006) described four different phases for the development of tryptic activity capacity. In this ‘four-phase model’ the increasing tryptic enzyme capacity is intermitted by a critical phase with decreasing tryptic enzyme activity and finally tryptic enzyme activity decreases at the onset of metamorphosis when trypsin is functionally replaced by increasing peptic activity. Beside larval size and developmental state (age) tryptic enzyme activity is correlated with food ingestion, gut fullness, feeding time and nutritional composition of the food (MacKenzie et al., 1999; Ueberschär, 1995). Accordingly, diel variability in tryptic enzyme activity of early life stages is observed, related to food ingestion and/or circadian rhythm (Fujii et al., 2007; MacKenzie et al., 1999; Tillner et al., 2014; Ueberschar, 1995).

1.4 Approach to improve larval digestive capacity

Considering the ontogenetic changes of the larval digestive tract it became clear that larval digestive capacity is limited and therefore can lead to mismatches during larval rearing. Adequate feeding during the entire larval development is still challenging. One challenge is to overcome the periods of minor proteolytic capacity. An approach to overcome this problem is to find methods stimulating the production or secretion of digestive enzymes which are essential for larval digestion. This can be achieved by identifying substances increasing the production and secretion of trypsinogen and consequently facilitating an increasing tryptic enzyme activity. First achievements to increase the tryptic enzyme activity level of fish larvae without any additional trigger such as food ingestion is reported using a mixture of bovine serum albumin (BSA) and FAA and by using an extract of rotifers (Koven et al., 2002; Webb and Holt, n.d.). Knowledge about such substances can help to facilitate the application of MD. Although there are many progresses to improve the digestibility of MD, MD are harder to digest than live food. However, by adding these substances into MD as chemical stimuli, the digestibility and attractiveness of MD could be improved. Such substances could be compared with the ‘unconditional stimuli’ in the dog experiments from Pavlov. Pavlov described an unconditional stimuli (e.g. food) as something that trigger a natural occurring response (’unconditional response’). This would mean that unconditional stimuli such as a mixture of FAA and BSA (Koven et al., 2002) or rotifer extract (Webb and Holt, n.d.) trigger the natural occurring response of tryptic enzyme activity in fish larvae.

1.5 Aim of this study

This study was designed to determine whether chemical / natural substances stimulate the secretion of trypsinogen and consequently facilitate tryptic enzyme activity. European sea bass larvae were chosen as model organism for these experiments, because of their importance in aquaculture due to a growing industry in the last decades, particularly in the Mediterranean (STECF, 2013). Useful for the interpretation of the trigger experiment is a general overview about the diel variability of tryptic enzyme activity of sea bass larvae and the tryptic enzyme capacity at different age classes. Accordingly, before examining whether various triggers can stimulate the trypsinogen secretion, the general diel variability of the tryptic enzyme activity of sea bass larvae was studied at 13 dph, 20 dph and 27 dph. This experiment should examine

(I) if the tryptic enzyme activity level changes during the day (diel rhythm) while comparing fed and unfed larvae;

(II) if there is a change in the diel rhythm of tryptic enzyme activity with age.

The main focus in this study is to stimulate the trypsinogen secretion of European sea bass larvae, with different triggering substances. Various studies already have identified single FAA (L-isomers) and mixtures of FAA as feed attractant and reported positive effects on feeding behavior for juveniles and fish larvae (Goh and Tamura, 1980; Kolkovski et al., 1997a; Mackie et al., 1980). It is known that prey organisms (zooplankton) release large amounts of FAA and other organic substances which are categorized as potential feed attractants. Kolkovski et al. (1997a) reported an improvement in MD feeding rates of sea bream larvae due to the chemical stimulation of *Artemia* rearing water. Further Kolkovski et al. (1997a) could attribute this success to four metabolites of the rearing water: betaine, arginine, alanine and glycine. Recent experiments examined the effect of different FAA mixtures on the swimming behavior of sea bass larvae, thereby a strong response was found by exposing the larvae to a mixture of glycine and betaine (Sommerfeld, 2014). Furthermore several studies reported positive effects in feeding behavior of different fish species (larvae – adult) due to supplementation of hydrolysates / extracts of marine organisms (Kolkovski and Tandler, 2000; Kolkovski, 2008; Kolkovski et al., 2000; Tandler et al., 1982). Although various studies examined the effect of these substances on feeding behavior, few studies exist which investigated the biochemical effect of these substances on digestive enzymes of fish larvae. Based on these observations two mixtures of FAA (BAAG – betaine-alanine-arginine-glycine, GB – glycine-betaine) and two ‘extracts’ (MY – Mytilus edulis, ARW – *Artemia* rearing water) were chosen for this study as possible substances to trigger the secretion of trypsinogen. This experiment should examine

(I) if larvae, which are exposed to one of the four different trigger solutions (ARW, BAAG, GB, MY), have a higher tryptic enzyme activity compared to larvae without any trigger;

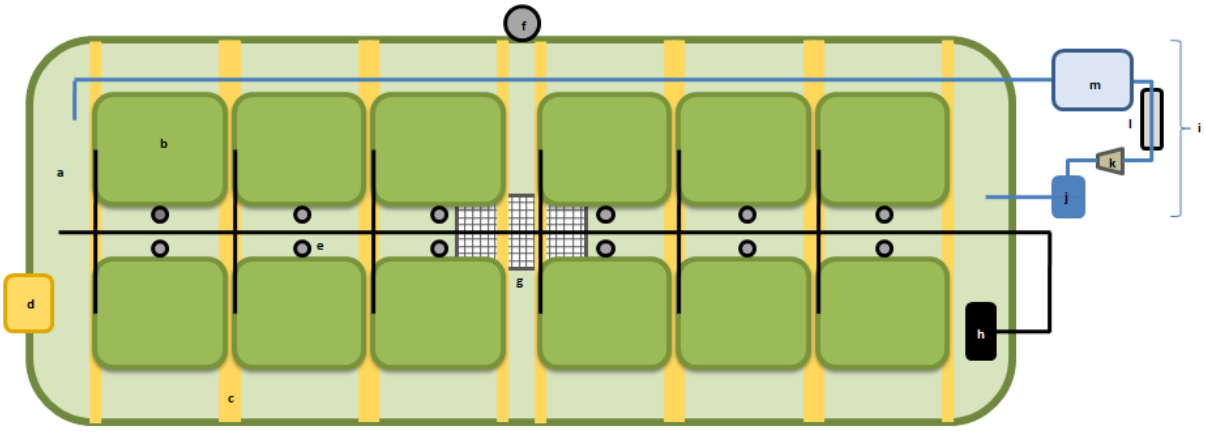
(II) if the effect of the different trigger solutions on tryptic enzyme activity changes with age.

2 Materials and Methods

2.1 Rearing conditions

2.1.1 Rearing system

Larval rearing was conducted in a specialized system for larval fish rearing. This system consists of two separate, re-circulating water units having 12 green conical tanks for larval rearing (each tank approx. 60 – 70 L water volume) and one large ambient tank (approx. 6000 L water volume). For this study, one unit was used for larval rearing and the experiments were conducted in the second unit (Figure 2). The large volume of water in the ambient tank helps to stabilize the temperature in the 12 rearing tanks. A submersible pump circulates seawater from the ambient tank into the rearing tanks and back through a standpipe system into the ambient tank. The water volume in the ambient tank circulates continuously through a UV-sterilizer, a filter cartridge (20 µm) and a cooling system. Within the ambient tank, the water inlet and outlet are in opposite corners of the tank. A protein skimmer (Aqua Medic) is fixed at the edge of the ambient tank to remove organic compounds. An automatic dimmer (Light-Control GLC) emulates day, twilight and dawn periods using halogen light for twilight / dawn simulations by gradual dimming and permanent fluorescent lighting simulates daytime (max. 450 lux).



a	-	**ambient tank** (approx. 6000 L water volume)	**h**	-	**submersible pump** (circulation between ambient tank and small tanks)
b	-	**small conic tank** (approx. 60 L water volume)	**i**	-	**cooling / filtration circulation**
c	-	**wooden beams** to carry small conic tanks	**j**	-	**pump**
d	-	**protein skimmer**	**k**	-	**filter cartridge** (20µm)
e	-	**standpipe** (small conic tank)	**l**	-	**UV-sterilizer**
f	-	**standpipe** (ambient tank)	**m**	-	**cooling system**
g	-	**drain grate** (ambient tank)			

Figure 2: Outline of the larval rearing system / experimental system

Each of the 12 rearing tanks has a peripheral water inlet and a central outlet. The peripheral inlet forms a slight current within each tank (Figure 3). In each rearing tank a pipe, covered by a 300 µm gaze, is fixed over the central outlet to avoid loss of larvae into the ambient tank. An air tube for gentle aeration is centrally arranged on the bottom in each small tank. The water flow and the aeration can be regulated for each of the 12 tanks individually. A surface protein skimmer could be immediately installed in each tank if needed to clean the water surface from dirt or oily sheen (e.g. remains of feeding). This skimmer was particularly important when larvae began inflating their swim bladder at the water surface.

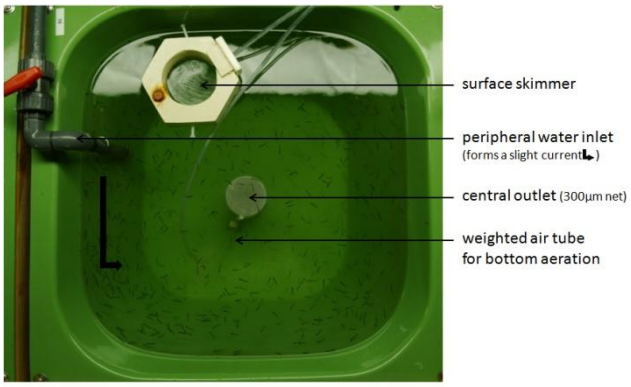


Figure 3: Tank for larval rearing. Peripheral water inlet forms a slight circulating water movement in the tank.

The rearing system was initially filled with sand-filtered, UV-sterilized and ozone treated North Sea seawater from storage tanks. Sea water was adjusted to the required salinity for larval rearing and aerated for at least 12 hours in an external tank to reduce ozone concentration. Water exchange was accomplished with the same procedure when water quality (ammonia concentration) reached limits for larval rearing.

2.1.2 Breeding of *Artemia* (spp.) nauplii

After mouth opening, sea bass larvae were fed by Micro *Artemia* nauplii (Micro *Artemia* Cysts 430, Ocean Nutrition; *Artemia* instar I nauplii: ± 430 µm). *Artemia* nauplii were cultured by incubating *Artemia* cysts in cylindrical translucent 18-L tanks with a conically tapered bottom and a drain cock (Figure 4). The tanks were filled with filtered and UV treated North Sea water (27 – 30 PSU). Tanks were continuously aerated, illuminated and temperature was kept at 27°C during incubation. The maximum cyst density was approximately 2 g Cysts L^{-1} (corresponding to about 300.000 hatched *Artemia* nauplii L^{-1}). A six-step procedure was used to breed and clean *Artemia* nauplii to the proper stage to feed them to the sea bass larvae (Figure 4). After 24 hours of incubation (step 1, Figure 4), *Artemia* instar I nauplii could be harvested. Before harvesting, the *Artemia* nauplii were separated

from the empty or unhatched cysts (step 2, Figure 4). For separation the aeration and normal illumination was turned off and an external light source was attached to the bottom of the tank. After 10 – 15 minutes *Artemia* nauplii (phototaxis) aggregated at the bottom of the tank, while unhatched cysts floated in the upper part of the tank. The aggregated *Artemia* nauplii were drained through the drain cock at the bottom of the tank and collected in a fine sieve (step 3, Figure 4). The nauplii were washed (step 4, Figure 4), concentrated and decanted into a 1 L beaker. An external light at the bottom of the beaker was used to separate nauplii and from remnants of hatched cysts or unhatched cyst a second time (step 5, Figure 4). Remnants were siphoned from the surface. *Artemia* nauplii were counted and subdivided into three equal portions (step 6, Figure 4). One portion was fed directly to the sea bass larvae in the morning, while the other two portions were stored on ice and fed to the sea bass larvae later in the day (in the early afternoon and late evening).

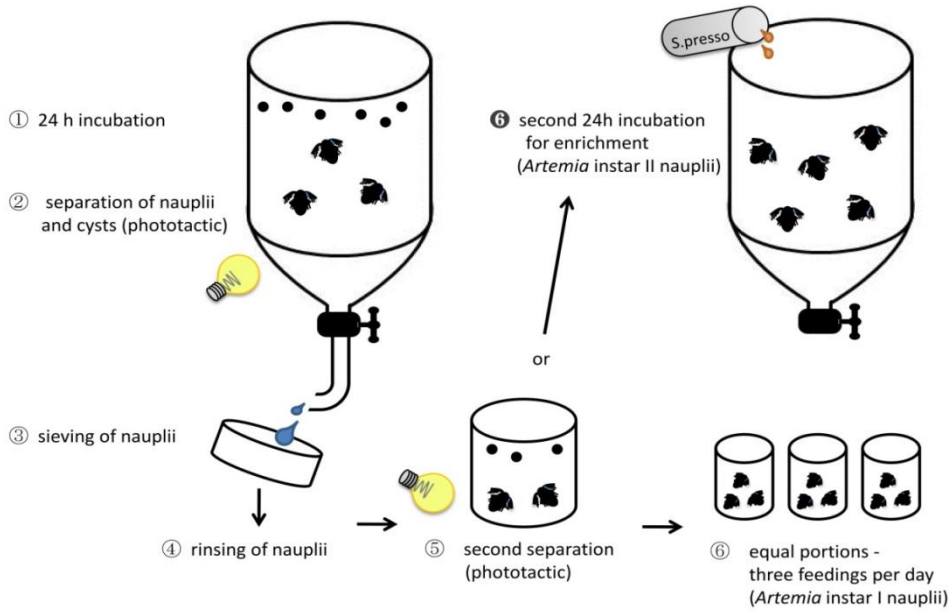


Figure 4: Work process to breed and harvest *Artemia* nauplii**.** *Artemia* nauplii were incubated for 24h (instar I) and fed to the larvae until 26 / 27 days post-hatch (dph) or nauplii were incubated for 48h with additional enrichment (instar II) and were fed to the larvae from 26 / 27 dph on.

Sea bass larvae until the age of 26 days post hatch (dph) were fed with *Artemia* instar I nauplii, older larvae were fed by enriched *Artemia* instar II nauplii. For rearing of *Artemia* instar II nauplii, instar I nauplii were reared another 24 hours (alternative step 6, Figure 4) under same conditions and were additionally fed with a liquid enrichment (S.presso, INVE). *Artemia* instar II nauplii were harvested, subdivided and stored or fed directly to the larvae like *Artemia* instar I nauplii.

2.1.3 Larval rearing

Sea bass larvae at the age of 3 dph were collected from a commercial hatchery (Ecloserie Marine de Gravelines) in Gravelines, France. Within ten hours, the larvae were transported in sea water under cooled (approx. 14 °C) and oxygenated conditions, to the facility of the Gesellschaft für Marine Aquakultur mbH (GMA) in Büsum, Germany. The larvae were homogenously distributed in the 12 rearing tanks (approx. 150 larvae L^{-1}). The larvae were reared in darkness until spot-check testing revealed that most of the larvae had opened their mouth (7 dph) and absorbed most of their yolk sac ('French method'). Rearing in darkness reduces larval activity in the first days after hatching, thus reducing energy demand for swimming and so increasing larval growth and yielding a larger mouth diameter. This concept is beneficial especially when *Artemia* nauplii are used as first food without co-feeding of other (smaller) live food organisms.

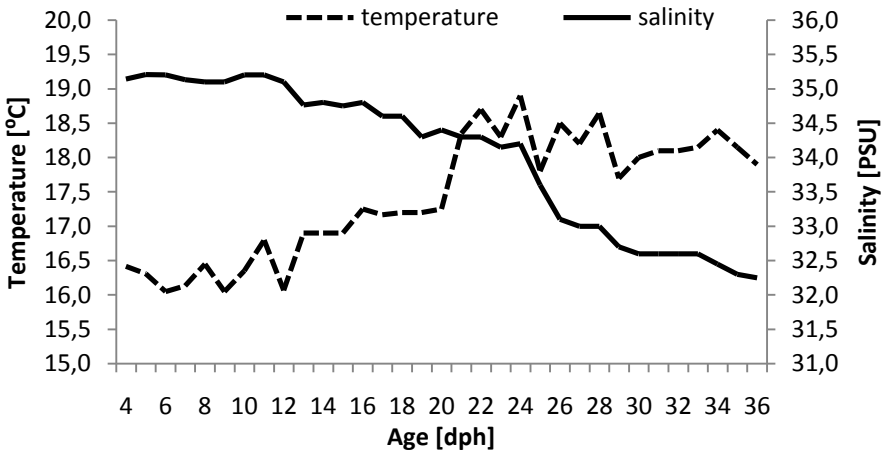


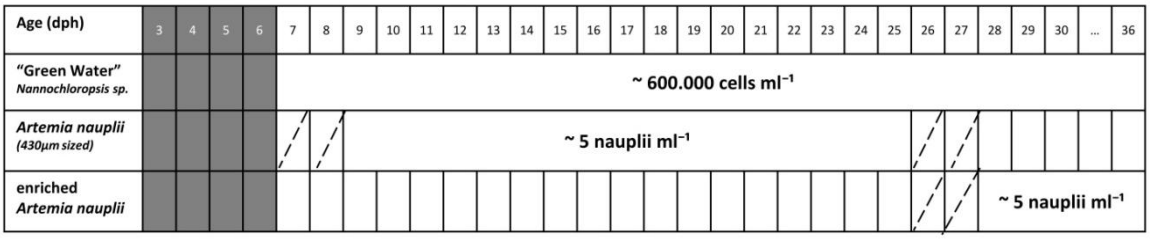
Figure 5: Temperature and salinity variations during larval rearing**.** Temperature slowly increased (max. ≤ 0.5°C day^{-1}), salinity gradually reduced.

In accordance with rearing protocols for sea bass larvae (Moretti et al., 1999; pers. comm., S. Zeytin) water parameter and the function of the system were set, adjusted and checked daily. Water flow was set at a gentle level at 4 dph and was carefully increased until the end of rearing (max: 1 L min^{-1}). Throughout the rearing process water temperature was gradually (max. ≤ 0.5°C day^{-1}) increased from 16°C to 18°C and salinity was gradually reduced from 35 PSU to 32 PSU (Figure 5). Aeration was adjusted to yield very slow coarse bubbling. The oxygen saturation ranged between 85 % and 100 %. The ammonia concentration remained at 0 to < 0.2 mg NH_4 L^{-1} until 14 dph and was between 0 to 0.8 mg NH_4 L^{-1} between 14 dph and 36 dph. To avoid high ammonia concentrations (> 0.2 mg NH_4 L^{-1}) a daily water exchange from the ambient tank (about 800 L) was accomplished. The nitrite concentration

remained at a low level (0 – 0.1 mg NO_2 L^{-1}). The bottom of the rearing tanks was siphoned several times during the day to remove detritus.

At ages of 7 dph and 8 dph, fluorescent light were turned on for 4 h and 14 h, respectively to spot-check if larvae started feeding on *Artemia* nauplii (Micro *Artemia* Cysts 430, Ocean Nutrition). From that time on, each day 1 – 2 ml of a concentrate of the microalgae *Nannochloropsi* ssp. (BlueBioTech) was added to each of the rearing tanks to provide a concentration of about 600.000 cells ml^{-1} (turbidity: approx. 2.5 NTU). Microalgae ('green water technique') were used being assumed to be beneficial in larval growth, survival and nutrition (Bengston et al., 1999; Cahu et al., 1998; van der Meeren et al., 2007). At the time of first feeding and addition of microalgae surface skimmer were installed into the rearing tanks. Starting at 9 dph, a 16 h photoperiod was used between 07:00 and 23:00 (16 h: 8 h light regime) including 15 minutes twilight / dawn period for sunrise and sunset simulation. The light intensity ranged between 200 to 450 lux at the water surface of the rearing tanks.

Table 1: Feeding regime for European sea bass larvae from 3 – 36 dph. Larvae were kept in darkness ("French method") from 3 dph to 6 dph. At 7 / 8 dph first feeding tests with *Artemia* nauplii (instar I). From 9 dph on, a daily regular feeding regime was implemented.



From 9 dph on, larvae were fed with *Artemia* nauplii at 08:00, 14:30 and 21:00 each day (Table 1). In the first three weeks the larvae were fed with *Artemia* instar I nauplii until 26 dph, afterwards the larvae were fed with enriched *Artemia* instar II nauplii. At 26 dph and 27 dph the larvae were fed with *Artemia* instar I nauplii and instar II nauplii to adapt the larvae to the new prey size.

2.2 Diel rhythm experiment

In order to evaluate whether larvae have an endogenous, diel rhythm in tryptic activity, measurements were made on fed (feedings: 08:00, 14:30 and 21:00) and unfed (without any food during the experiment) larvae sampled over a 24-h period (Figure 6). The experiments were conducted three times, when larvae were 13 dph, 20 dph and 27 dph to examine if the endogenous diel rhythm in tryptic activity is changing with age (larval development). The experiments started in darkness at 06:30 am by transferring 300 larvae from the rearing tanks

into one tank of the experimental system with similar water parameters but without "green water" (*Nannochloropsis* sp.). This group of larvae was left unfed. Sampling took place at 06:30 (before sunrise), 07:15 (after sunrise) and from 8:00 till 23:00 (after sunset) every hour and on the next morning at 06:30 (before sunrise). For each time of sampling ten larvae from the fed larvae and from the unfed larvae were sampled with a soft pipette. Each group was stored separately in Eppendorf tubes. The tubes were immediately frozen at - 80°C.

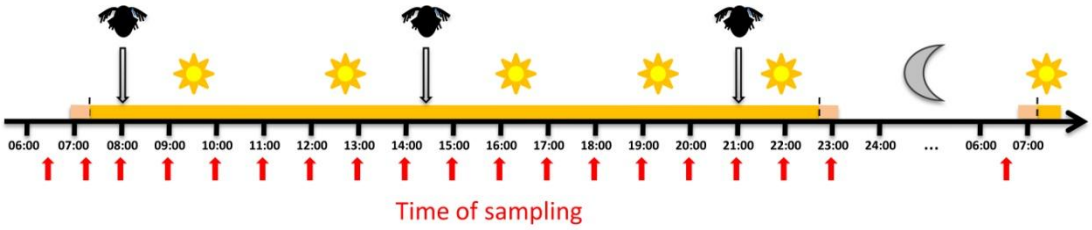


Figure 6: Time schedule of sampling during diel rhythm experiment**.** Time scale shows the 16 h – lighting period and the dawn / twilight period. The black *Artemia* nauplii symbolize the feeding time at 8:00, 14:30 and 21:00. Red arrows represent the time of sampling.

2.3 Trigger experiment of trypsinogen secretion

2.3.1 Preparation of trigger solutions

Two combinations of amino acids (BAAG: betaine - alanine - arginine - glycine and GB: glycine - betaine) and two natural extracts (MY: blue mussel extract (*Mytilus edulis.*) and ARW: *Artemia* rearing water) were used as 'trigger' in the experiment to stimulate the secretion of trypsinogen (Table 2). The preparation of amino acid mixtures and the associated experiment took place at the same day. The preparations of the natural extracts took place one / several day(s) before the experiment. All the preparation work was conducted at cold temperature to avoid a decline in the quality of the trigger solution by degradation of e.g. amino acids. Cold water was used for the preparation of the solutions; frozen materials / liquids were thawed on ice or in a refrigerator and all dilutions or extracts were stored on ice or in a refrigerator until used for the experiment.

Table 2: Trigger solutions for the trigger experiment to stimulate the trypsinogen secretion. List of components and concentrations of the trigger solutions used in the 60 L volume of the experimental tank. * based on Kolkovski et al. (1997)

Trigger solution	Type	Composition	Concentration
ARW	natural extract	rearing water of *Artemia* spp.	2 L of *Artemia* rearing water (~375 nauplii ml^{-1}) in 60 L tank volume *****
BAAG	mixture of amino acids	Betaine, Alanine, Arginine, Glycine	5.3 ng/ml betaine, 5.9 ng/ml alanine, 0.2 ng/ml arginine, 9.1 ng/ml glycine *****
GB	mixture of amino acids	Glycine, Betaine	10^{-4} M
MY	natural extract	Extract of blue mussels (*Mytilus edulis*)	10^{-2} g/l

ARW – *Artemia* rearing water

Artemia cysts were incubated for 24 hours (see also "2.1.2 Breeding of *Artemia* nauplii*"*) before the experiment. The salinity of *Artemia* rearing water was adjusted to the salinity of the tanks used in the experiment. Prior to the trigger experiment the trigger solution was prepared by sieving the *Artemia* nauplii from the rearing water, followed by three filtering processes (filter papers MN 615, Macherey-Nagel) to ensure that no nauplii, cysts or other particles are left in the trigger solution. The test concentration was adjusted after Kolkovski et al. (1997a). Kolkovski et al. (1997a) kept sea bream larvae in a 600 ml beaker and added *Artemia* rearing water with an incubation density of 12 nauplii ml^{-1}. The incubation density of *Artemia* nauplii in this study was on average 375 nauplii ml^{-1}. Therefore about 2 L from the rearing water were added to the experimental tank (60 L) to simulate the concentration of potentially triggering components of *Artemia* rearing medium with an incubation density of 12 nauplii ml^{-1}.

Mixtures of amino acids: BAAG and GB

The test concentration of the BAAG mixture in the experimental tanks was chosen following the analysis results of *Artemia* rearing medium (5.3 ng ml^{-1} betaine, 5.9 ng ml^{-1} alanine, 0.2 ng ml^{-1} arginine, 9.1 ng ml^{-1} glycine) from Kolkovski et al. (1997a). The test concentration of the GB mixture in the experimental tank (60 L) should be about 10^{-4} M. Three hours prior to the start of the experiment the amino acids were mixed (~ 5 min mixing) with distilled water and if necessary adjusted to the desired concentration with seawater. Both solutions (BAAG, GB) were filtered (filter papers MN 615, Macherey-Nagel) to ensure that no solids are left. Exact adjustment of the salinity of the trigger solutions was not needed, because the added amount (100 ml) was negligible and the trigger solution was homogenously mixed in the water of the experiment tank before beginning of the experiment.

MY – extract of blue mussels (*Mytilus edulis*)

Blue mussels (*Mytilus edulis*) were used for the production of the extract. Living mussels were frozen at - 80°C. The test concentration of the extract should be about 10^{-2} g L^{-1} (Kolkovski, S., 2008) in the experimental tank. Therefore 60 g thawed offal and flesh of blue mussel were homogenized with a blender and mixed with 1 L seawater. This solution was filtered several times (filter papers MN 615, Macherey-Nagel) until solid particles were removed and until the color of the extract became transparent to avoid any possible visual stimuli. The extract was equally portioned (10 ml) and frozen at - 80°C until the start of the experiments. Six hours prior to the start of the experiments three portions of the extract were unfrozen and each portion was mixed with 90 ml seawater. Again exact adjustment of the salinity of the trigger solution was not needed, because it was mixed in the tank water before the beginning of the experiment and the added amount (100 ml) was negligible compared to the tank water volume.

2.3.2 Experimental design

The aim of this experiment was to test if a chemical stimulus stimulates the trypsinogen secretion of European sea bass larvae. Larvae at ages of 14 / 15 dph, 21 / 22 dph and 28 / 29 dph were exposed to four different trigger solutions (BAAG, GB, ARW and MY; Table 2). The water of the experimental tanks had the same temperature and salinity as the water of the rearing tanks. Application of microalgae ("green water") was omitted to avoid possible effects from the algae on the trypsin activity. Trials of BAAG and ARW were carried out at 14 dph, 21 dph and 28 dph, GB and MY at 15 dph, 22 dph and 29 dph. BAAG and GB solutions were tested in the morning, ARW and MY in the afternoon.

At 06:30 am (before sunrise) of the day of each trial, 600 larvae were transferred from the rearing system into two storage tanks of the experimental system to acclimate the larvae. Transferring the larvae in darkness should minimize feeding activity. The larvae were kept in floating transparent Plexiglas tubes (20 cm diameter, 300 µm gaze at the bottom, adjustable immersion depth) in the storage tanks (2 L tank water volume) and adapted to the experimental system for 3-5 or 6-8 hours, depending if the trial started in the morning or in the afternoon.

For each trial six tanks were randomly selected from the system, three tanks to test one of the trigger solutions and three control tanks without any trigger solution. During the trials, water

circulation was stopped by closing the outlet and switching off the water inlet of the experimental tanks (Figure 7). Before the trial started the water volume of the six tanks was adjusted to 60 L. The testing of each trigger solution was separated in three consecutive experimental phases due to technical reasons (Figure 8). Neither it was possible to start the trial in six tanks simultaneously nor was it possible to carry out samplings in six tanks simultaneously. One experimental phase involves running the trial in one tank with a trigger solution and in one control tank. The start of the trials in the control tank was delayed by five minutes to allow an appropriate sampling procedure.

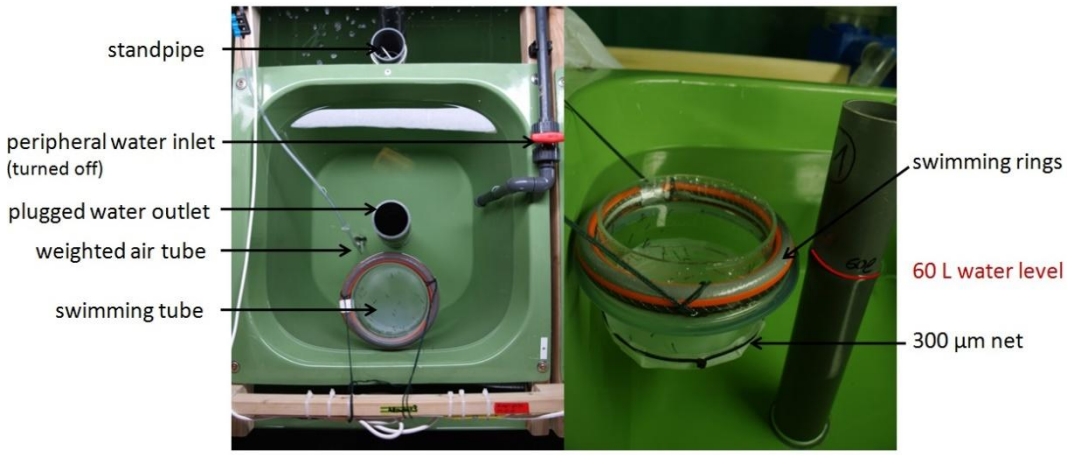


Figure 7: Experimental setup of the trigger experiment in one of the six experimental tanks.

The commencement of each experimental phase started by adding trigger solution into the tank water and mixing the water for two minutes before the trial started. Transparent floating Plexiglas tubes with a 300 µm gaze at the bottom kept the larvae in 1.5 L water volume of the tank during the trial (Figure 7). These tubes supported the sampling procedure in concentrating the larvae in a smaller volume inside the tanks and were helpful in reducing larval stress during sampling. Swimming tubes were introduced into the tanks after thoroughly mixing the trigger solution into the tank water. Preparation of the control tank in each experimental phase was conducted in the same way except of adding a trigger solution.

One trial started with transferring 40 - 50 larvae into the swimming tube. After 10, 20 and 30 min ten larvae were sampled and stored in Eppendorf tubes with a soft pipette. Tubes were kept on dry ice after sampling and subsequently stored at - 80°C. Once the last sampling (after 30 min) in the tank with the trigger solution and the control tank was carried out the first experimental phase ended and the preparation for the second phase was started immediately. After three phases the water from the experimental tanks was rinsed, tanks were cleaned with freshwater and filled up again with water from the experimental system.

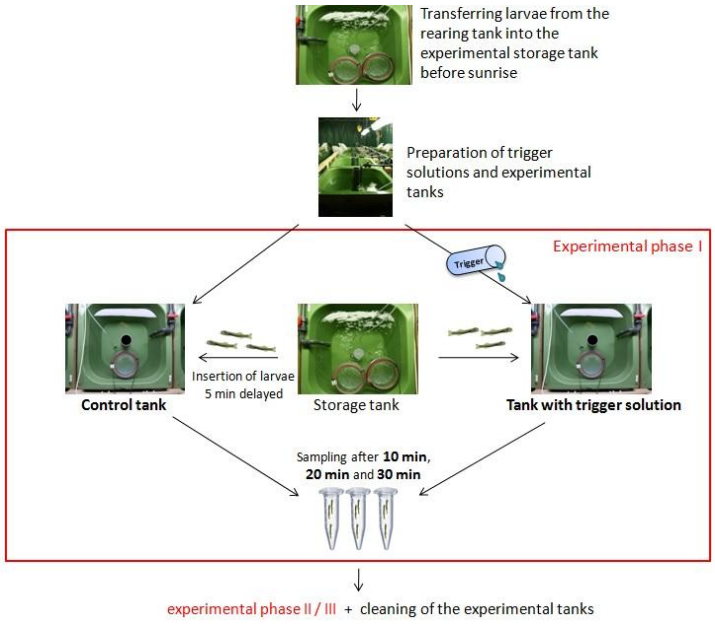


Figure 8: Experimental design of the trigger experiment to stimulate the trypsinogen secretion. The trial of each trigger solution (ARW, BAAG, GB and MY) was divided in three consecutive experimental phases. The trial in one control tank and one tank with a trigger solution started and was completed before the next experimental phase in the second control/trigger - tank started. During one phase the start of the trial in the control tank and the tank with the trigger solution was delayed about five minutes. Sampling took place after 10, 20 and 30 min after insertion of the larvae into the experimental tank (floating Plexiglas tube).

2.4 Lab analysis

2.4.1 Sample preparation

Samples of both diel rhythm and trigger experiments were prepared similarly. The preparation was done for all the larvae individually according to Ueberschär (1988). The samples were thawed on ice and gently flushed from the Eppendorf tubes into a cooled Petri dish with distilled water. In the Petri dish the larvae were rinsed again with cold distilled water. Gut fullness and total length (mm) was measured individually. Gut fullness was scored on a scale from 0 (empty) to 4 (full) according to MacKenzie et al. (1999). Each larva was homogenized in an Eppendorf tube with a micropistill in 125 µl TRIS buffer. The homogenate was centrifuged for 45 min with 4.000 x g at 0°C (Thermo Heraeus Multifuge X3R). After centrifugation the samples were stored on ice until analyzing the tryptic enzyme activity.

2.4.2 Analysis of tryptic enzyme activity

The analysis of the tryptic enzyme activity is based on a highly sensitive fluorescence assay according to Ueberschär (1988) with the modifications described by Ueberschär et al. (1992). This assay is based on the use of a specific synthetic substrate, which is linked with the amino acid arginine on a fluorescent (7-amino-4-methylcoumarin, MCA). Trypsin belongs to the

group of (serine) endopeptidase, which hydrolyses peptide bonds mainly after the position of basic amino acids and therefore splits the substrate and the fluorescent at the link position of arginine. The substrate-fluorescent complex already shows a faint fluorescence but the free fluorescent shows a much higher fluorescence. The more trypsin in the sample or in the trypsin standard dilution (for calibration curves) is active, the more of the substrate-fluorescent complex will be split and so the more of the fluorescent is released. The increase of fluorescence caused by released fluorescent can be measured (fluorometer) and can be converted into hydrolyzed fluorescence products (MCA) per time unit and larva via a linear regression obtained from a calibration curve of pure MCA (fluorescent). MCA calibration curve is formed by single-point fluorescence measurements at defined MCA concentrations (4.8 - 720 nmol MCA).

In the following chemicals used, preparation of standard / calibration curves and the measurement of the tryptic enzyme activity are described in more details.

TRIS-HCL buffer

TRIS-HCL buffer is needed for all the following preparations (substrate, trypsin standard curve, MCA calibration curve). The buffer is composed of Tris-(hydroxymethyl)-aminomethane ($C_4H_{11}NO_3$, MERCK) and Calciumchlorid-dihydrate ($CaCl_2$ x 2 H_2O, MERCK) mixed with distilled water (ELIX system). The pH of this buffer will be adjusted with hydrochloric acid (37 %, HCL) to a pH-value of 8.0.

Substrate

The substrate was Na-benzoyl-L-arginine-4-methyl-coumarinyl-7-amide (BZ-L-Arg-MCA, I-1070) from BACHEM in Heidelberg, Germany. The substrate was prepared with TRIS-HCL-buffer at a concentration of 0.2 mMol. Fresh substrate was prepared on daily basis when analyses were conducted.

Trypsin standard curve

The regular preparation of the trypsin standard curve over the time period of the tryptic activity analysis allows one to ascertain whether the substrate and the fluorometer operate consistently during the measurement period. Trypsin standard curves were generated using a dilution series of a stock solution of trypsin from bovine pancreas (SERVA, 37289) and albumin from bovine serum (SERVA, 11924). Trypsin and albumin were mixed at a ratio of 1:10 with TRIS-HCL buffer. This stock solution was diluted with TRIS-HCL buffer to a dilution series with the lowest concentration of 0.1 ng ml^{-1} trypsin and the highest

concentration of 10 ng ml^{-1} trypsin. For the fluorescence measurement 50 µl of each dilution and 250 µl substrate were pipetted into a microplate well. The measurement was performed with a microplate fluorometer (TECAN infinite M200) and the emission was measured after 2, 4, 6, 8 and 10 minutes (380 nm excitation, 440 nm emission).The measurement of three wells with 300 µl TRIS-HCL buffer was used as blank. The trypsin standard curve was always repeated whenever new buffer was prepared.

MCA calibration curve

This calibration curve is based on the dilution series of 7-amino-4-methylcoumarin (MCA, BACHEM, Q-1025). For the stock solution 2 mg MCA was dissolved in 400 µl dimethylsulfoxide (DMSO, C_2H_6OS, SERVA) and mixed with TRIS-HCL buffer. This stock solution was diluted with TRIS-HCL buffer to a dilution series with a minimum range of 4.8 nmol MCA to a maximum concentration of 720 nmol MCA. For the fluorescence measurement 300 µl of each dilution were pipetted into a microplate well. Three wells with 300 µl TRIS-HCL buffer were used as blank. Single-point fluorescence measurements of each dilution were performed with a microplate fluorometer at 30°C after 2, 4, 6, 8 and 10 minutes. Calibration curve were formed by calculating the mean of fluorescence over the five measurement points and plotting the results against the associated MCA concentration. The MCA calibration curve was always repeated with fresh TRIS-HCL buffer.

Measurement of tryptic enzyme activity

The measurement of the samples was carried out under the same conditions as the trypsin standard curve (TECAN infinite M200, 30°C, 380-440 nm). Tryptic enzyme activity of each sample (larva) was measured individually by pipetting 50 µl of supernatant of the centrifuged sample and 250 µl substrate into a microplate well. Three wells were filled with 50 µl TRIS-HCL buffer and 250 µl substrate as blank measurements. A maximum of 84 samples were measured simultaneously. The microplate was gently shaken and the temperature was adjusted to 30°C before the five fluorescence measurement cycles (after 2, 4, 6, 8 and 10 min) were started.

2.5 Calculation of individual enzyme activities

Values for tryptic enzyme activity were expressed as hydrolyzed fluorescence products (7-amino-4-methylcoumarin, MCA) per time unit and larva (nmol MCA min^{-1} $larva^{-1}$). First, the average of the changes in fluorescence over the five fluorescence measurement cycles is

calculated for each sample. Second, the mean of the changes in fluorescence of the buffer-substrate blank is subtracted from the mean fluorescence changes of each sample. Third, the dilution factor, meaning the amount of TRIS-HCL buffer in the sample homogenate is included in the calculation. Fourth, the values are divided by 2 to receive the results per minute, because the fluorescence measurement interval lasted for two minutes. The final step in calculating the amount of hydrolyzed MCA per time unit and larva was to substitute the result from the first four steps in the equation of the linear regression obtained from the MCA calibration curve.

2.6 Statistical analysis

Tryptic enzyme activity values in the diel rhythm experiment and the trigger experiment were tested for normality and homogeneity using the Shapiro-Wilk-test and the Levene's test. Differences in the tryptic enzyme activity among sampling time (06:30, 7:15, 8:00-23:00, 06:30 next day) for unfed or fed larvae during the diel rhythm experiment were tested with a Kruskal-Wallis one-way analysis of variance, in which time of sampling was treated as a categorical variable. If differences between sampling time were significant Mann-Whitney-U-tests with the *p*-value adjustment method after Benjamini and Hochberg (1995) were used for multiple comparison. A Kruskal-Wallis one-way analysis of variance, followed by Mann-Whitney-U-tests with the *p*-value adjustment method after Benjamini and Hochberg (1995) was also used to test differences in the tryptic enzyme activity among age (13 dph, 20 dph and 27 dph) for the unfed or fed larvae. Correlation coefficient between total length and tryptic enzyme activity of fed larvae were calculated with Kendall`s rank correlation. Mann-Whitney-U-test tested tryptic enzyme activity between unfed and fed larvae at each time of sampling. Differences in gut fullness among unfed and fed larvae were tested with a Fisher`s exact test with Monte Carlo simulation for each time of sampling. Correlation coefficient between gut fullness and tryptic enzyme activity for 13 dph, 20 dph and 27 dph were calculated with Kendall`s rank correlation.

Differences in the tryptic enzyme activity among treatment (control, one trigger solution: BAAG, ARW, GB or MY) at each time of sampling (10, 20 and 30 min) during the trigger experiment were tested with a nested (three-way) ANOVA (Analysis of variance), in which the replicate tanks for each treatment were considered as a third factor. If the interaction between treatment, sampling time and replicate tanks were significant, a two-way ANOVA was used for each measurement phase (one control tank, one trigger tank) to compare the

tryptic enzyme activity between the trigger larvae and the control larvae at each time of sampling. To compare the tryptic enzyme activity of each treatment (BAAG, ARW, GB, MY and associated controls) between the tested age groups (14 / 15 dph, 21 / 22 dph and 28 / 29 dph) a Kruskal-Wallis one-way analysis of variance was used, followed by Mann-Whitney-U-tests with the *p*-value adjustment method after Benjamini and Hochberg (1995). Differences were considered significant at $\alpha \leq 0.05$. Statistical analysis was performed by using R (3.1.2) for Windows.

3 Results

3.1 Larval development

At 3 dph typical body pigmentation for sea bass yolk-sac larvae of yellow and black chromatophores developed (Figure 9 a), particularly ventral along the body contours, dorsal in patches, in front of the eyes and at the yolk-sac (Russel, 1976). The yolk-sac larvae had a primordial fin and lacked of a functional mouth and eye pigments (Figure 9 a). At 6 dph random inspection revealed several larvae with partially developed mouth opening. At time of first feeding (7 dph) most of the larvae opened their mouth and eye pigmentation was completed. Random inspection of gut fullness revealed that about 20 % of the larvae started feeding. At 10 dph 40 % of the larvae had a filled gut, while most of the yolk sac was absorbed. Initial inflation of the swim bladder was recognizable at 11 dph. At 12 dph 90 % of the larvae revealed filled guts. From 13 dph to 17 dph increased mortality was observed. From 20 dph on, typical developmental changes of the body pigmentation was observed, meaning the general coloring changed to grey / green colors, the ventral pigmentation intensified and the dorsal pigmentation reduced to the postanal body part. At 23 dph the flexion of the notochord and advanced reduction of the primordial fin was recognizable. A few larvae with a deformation at the beginning of the caudal fin were observed between 24 – 30 dph (Figure 9 c / d). During the entire rearing period (3 – 40 dph) approx. 30 % of initially 100.000 larvae were used for various experiments and after 4 weeks of rearing and experiments approx. 9.000 larvae remained.

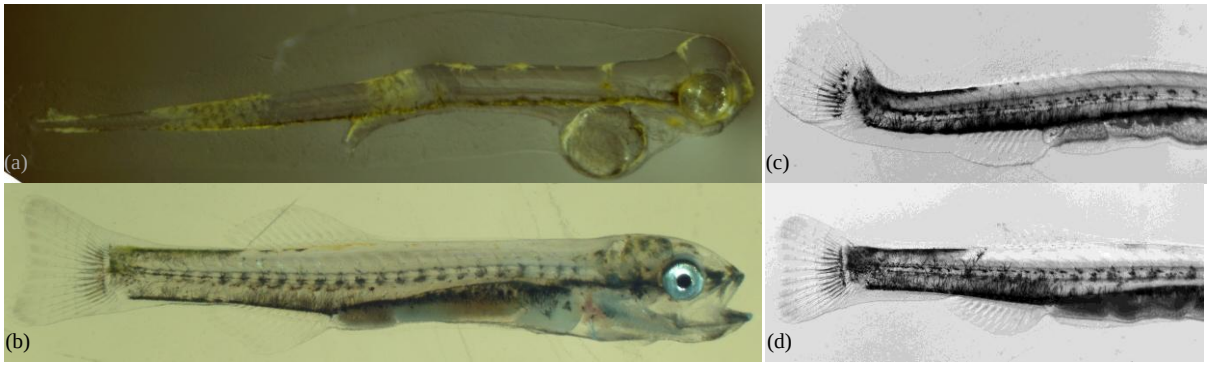


Figure 9: European sea bass larvae at different developmental stages. (a) Larva at 3 dph with yolk sac. (b) Larva at 37 dph, structures like fins, organs etc. are individually recognizable. (c) Deformation at the beginning of the caudal fin for a few larvae at 24 – 30 dph. For comparison: postflexion larva without deformation (d).

3.2 Diel rhythm experiment

3.2.1 Trial at 13 dph

At 13 dph the tryptic enzyme activity (hydrolysed MCA nmol min^{-1} $larva^{-1}$; mean ± standard deviation (SD)) of fed larvae increased slightly during the day, from 0.79 (±0.26) at 06:30 to a maximum of 2.82 (±1.0) at 22:00 (Figure 10 a). In confirmation, significant differences in tryptic enzyme activity of fed larvae between the times of sampling were found ($p < 0.05$). A slight tendency of decreasing tryptic enzyme activity of unfed larvae during the day was observed. Accept the outlying mean tryptic enzyme activity at 10:00, tryptic enzyme activity of unfed larvae fluctuated only on a minimal activity level. However these fluctuation movements are comparable with the higher level fluctuation movements of fed larvae. Significant differences of tryptic enzyme activity of unfed larvae during the day mainly resulted from single outlying mean tryptic activity values, for instance at 10:00 (Figure 10). Significant differences in the tryptic activity between fed and unfed larvae were measured at 18:00 and from 20:00 to 23:00 ($p < 0.05$).

The gut fullness index (mean ± SD) of fed larvae increased from 0.34 (±0.52) to 4.0 (±0.01) from 06:30 to 23:00 (Figure 10 b). Gut fullness index was periodically lower during the day (e.g. between 10:00, 12 – 13:00, 16 – 17:00 and 20:00). Unfed larvae had partially filled guts, particularly at the beginning of the experiment from 06:30 until 11:00 (0.36 ± 0.35). A few differences in gut fullness index between fed and unfed larvae were detected from 12:00 to 23:00 ($p < 0.05$). Correlation between gut fullness index and tryptic enzyme activity resulted in $\tau = 0.41$ (Figure 10 c).

3.2.2 Trial at 20 dph

Over the time of the experiment at 20 dph, tryptic enzyme activity (hydrolysed MCA nmol min^{-1} $larva^{-1}$) of fed larvae increased significantly during the day ($p < 0.05$). Particularly from 15:00 until 23:00 an increase in tryptic enzyme activity could be observed, with a mean (± SD) maximum of 7.22 (±1.91) at 23:00 (Figure 11 a). A slight tendency of decreasing tryptic enzyme activity of unfed larvae during the day could be observed. Significant differences in tryptic enzyme activity of unfed larvae between the times of sampling were found with global test statistic (Kruskal-Wallis), but a pairwise post hoc statistic revealed no differences. Comparing the tryptic enzyme activity of fed and unfed larvae, unfed larvae showed a fairly

constant tryptic activity level over the whole day. Accordingly, tryptic enzyme activity was significantly different between fed and unfed larvae from 15:00 to 23:00 ($p < 0.05$).

The mean (± SD) gut fullness index of fed larvae increased from 0.83 (±0.41) to 3.34 (±0.82) from 06:30 to 23:00, without any considerable fluctuations (Figure 11 b). Unfed larvae showed partially filled guts, particularly at the beginning of the experiment from 06:30 until 12:00 (0.67 ± 0.24) and from 16:00 to 19:00 (0.21 ± 0.09). From 10:00 to 23:00 differences in gut fullness index between fed and unfed larvae were found ($p < 0.05$). Correlation between gut fullness index and tryptic enzyme activity resulted in $\tau = 0.58$ (Figure 11 c).

3.2.3 Trial at 27 dph

Tryptic enzyme activity (hydrolysed MCA nmol min^{-1} $larva^{-1}$; mean ± SD) of fed larvae at 27 dph increased from 06:30 (1.5 ± 0.41) to 23:00 (17.76 ± 6.94), with a temporary decline from 20:00 to 22:00 (Figure 12 a). In confirmation, significant differences in tryptic enzyme activity of fed larvae between the times of sampling were found ($p < 0.05$). Tryptic enzyme activity of unfed larvae decreased slightly during the day. However, accept the outlying mean tryptic activity at 06:30 the tryptic enzyme activity of unfed larvae fluctuated only on a minimal tryptic activity level. Significant differences of tryptic enzyme activity of unfed larvae during the day mainly resulted from single outlying mean tryptic activity values, for instance at 06:30. Consequently, tryptic enzyme activity was significantly different between fed and unfed larvae at times of the day between 09:00 and 23:00 ($p < 0.05$). Additionally, tryptic enzyme activity of unfed larvae was significantly higher at first sampling (06:30) than tryptic activity of fed larvae.

The increase of gut fullness index of fed larvae from 06:30 (0.17 ± 0.41) to 23:00 (3.86 ± 0.41) was intermitted by a fairly steady decrease from 16:00 to 21:00 (0.84 ± 0.41). Unfed larvae showed partially filled guts, particularly at the beginning of the experiment from 06:30 until 12:00 (0.29 ± 0.21) and again at 15:00 (Figure 12 b). Differences in gut fullness index between fed and unfed larvae were found from 09:00 to 23:00 ($p < 0.05$). Correlation between gut fullness index and tryptic enzyme activity resulted in $\tau = 0.64$ (Figure 12 c).

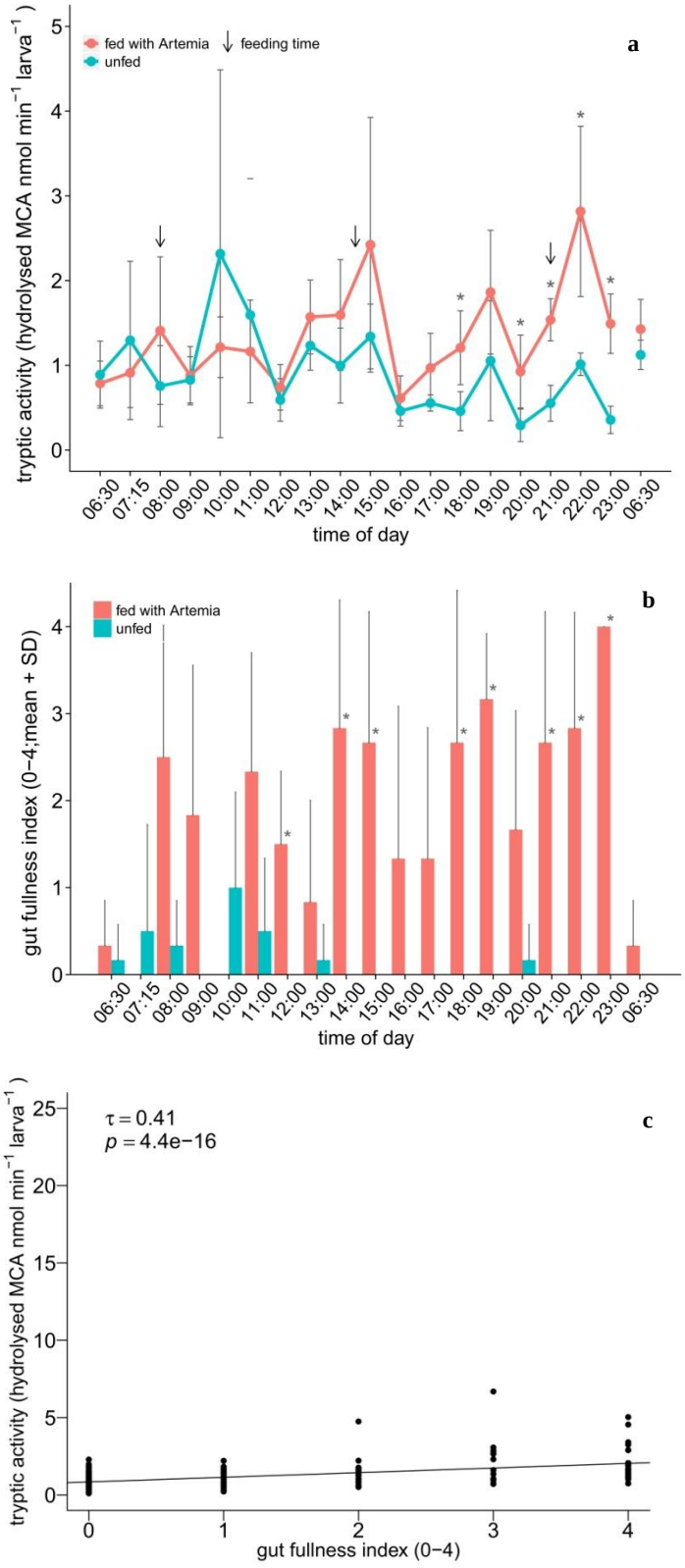


Figure 10: Tryptic enzyme activity (a), gut fullness index (b) and correlation (c) between tryptic enzyme activity and gut fullness index of sea bass larvae during the diel rhythm experiment at 13 dph. Sampling of unfed and fed larvae took place at 06:30, 07:15, from 08:00 to 23:00 every hour and 06:30 next day. Red line / bars represent the group of larvae fed three times during the experiment. Black arrows indicate feeding time. Blue line / bars represent unfed larvae. Values of a and b are means and standard deviations of six larvae. From 14:00 on, mainly no food particles (*Artemia*) were found in the gut of unfed larvae. Correlation included data of unfed and fed larvae.

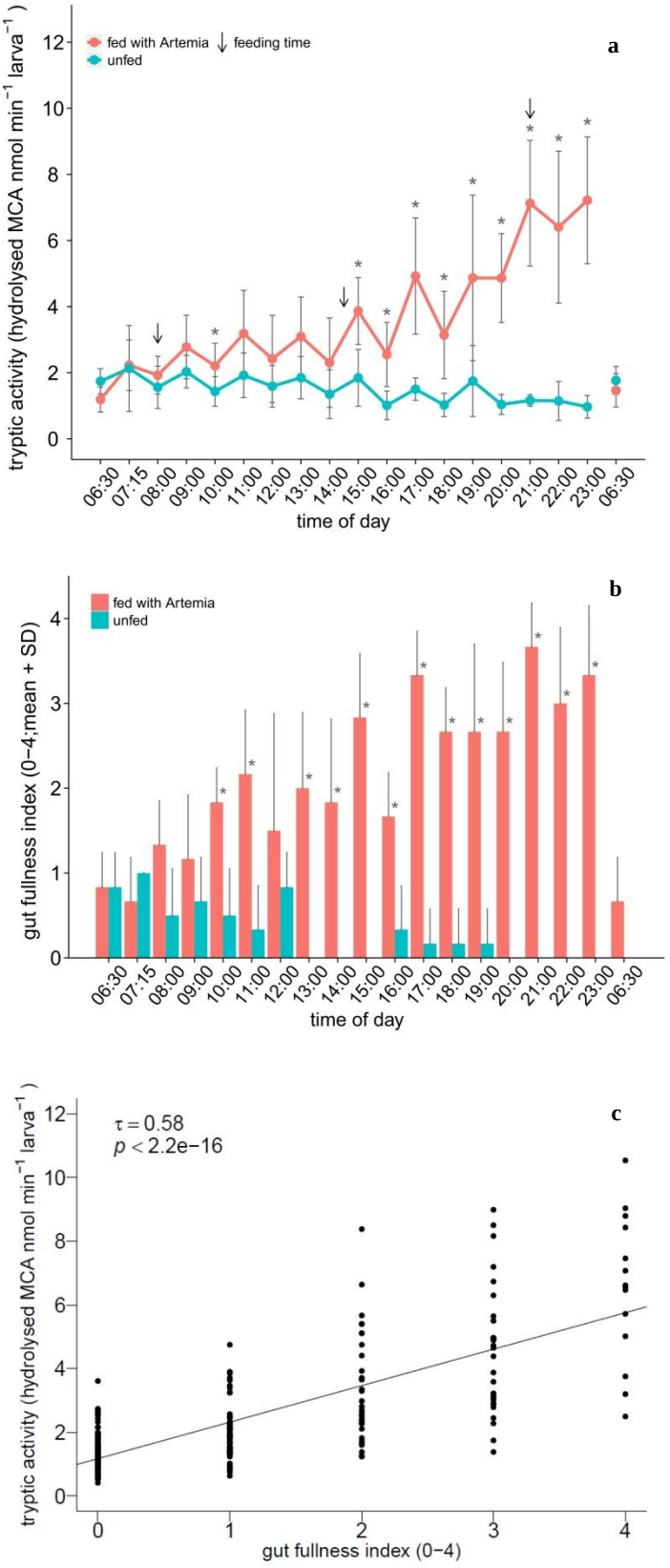


Figure 11: Tryptic enzyme activity (a), gut fullness index (b) and correlation (c) between tryptic enzyme activity and gut fullness index of sea bass larvae during the diel rhythm experiment at 20 dph. Sampling of unfed and fed larvae took place at 06:30, 07:15, from 08:00 to 23:00 every hour and 06:30 next day. Red line / bars represent the group of larvae fed three times during the experiment. Black arrows indicate feeding time. Blue line / bars represent unfed larvae. Values of a and b are means and standard deviations of six larvae. From 13:00 on, mainly no food particles (*Artemia*) were found in the gut of unfed larvae. Correlation included data of unfed and fed larvae.

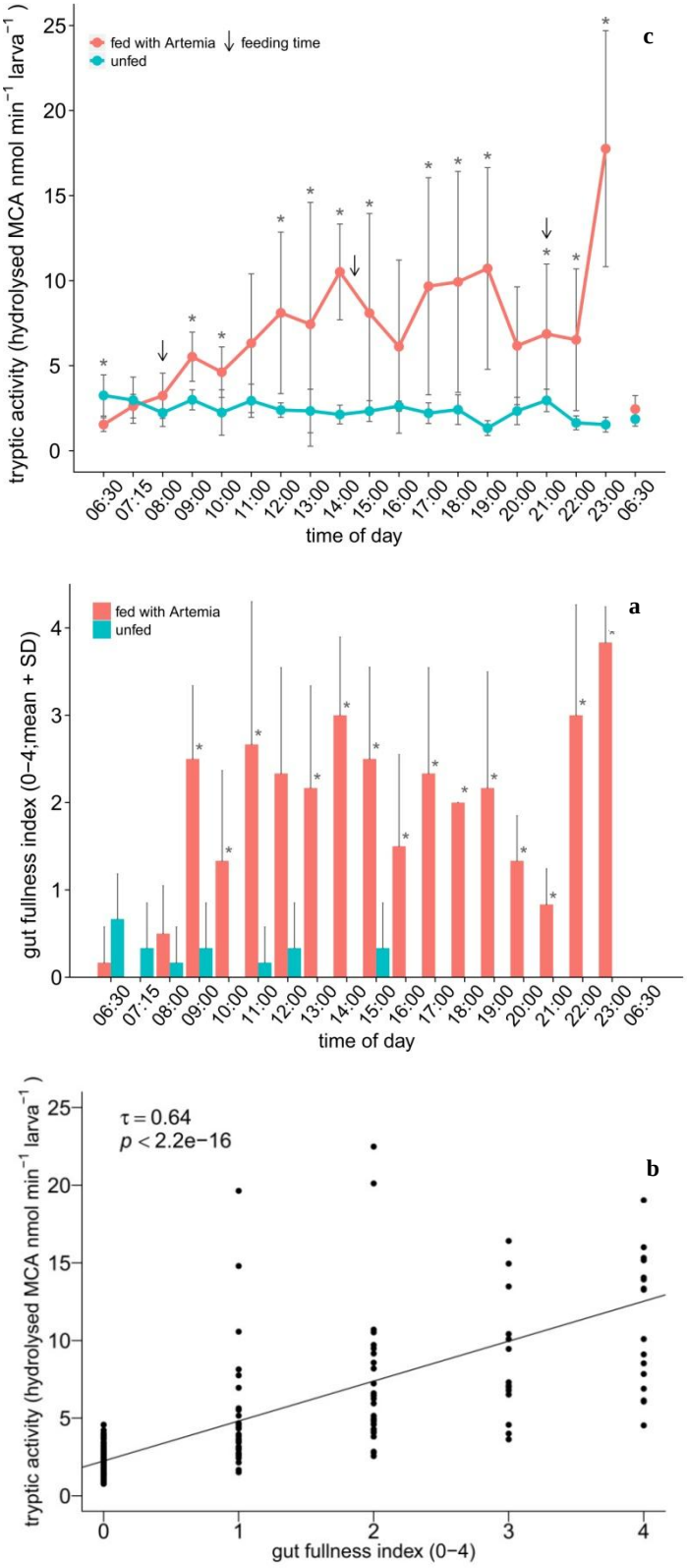


Figure 12: Tryptic enzyme activity (a), gut fullness index (b) and correlation (c) between tryptic enzyme activity and gut fullness index of sea bass larvae during the diel rhythm experiment at 27 dph. Sampling of unfed and fed larvae took place at 06:30, 07:15, from 08:00 to 23:00 every hour and 06:30 next day. Red line / bars represent the group of larvae fed three times during the experiment. Black arrows indicate feeding time. Blue line / bars represent unfed larvae. Values of a and b are means and standard deviations of six larvae. From 13:00 on, mainly no food particles (*Artemia*) were found in the gut of unfed larvae. Correlation included data of unfed and fed larvae.

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3.2.4 Comparison among age classes

Tryptic enzyme activity level of the unfed larvae increased with age ($p < 0.05$), but the extent of tryptic activity fluctuations during the day are similar for the different age classes, except for a few outlying values (Figure 13 b). The daily mean (±SD) tryptic enzyme activity (hydrolysed MCA nmol min^{-1} $larva^{-1}$) of unfed larvae was 0.93 (±0.82) at 13 dph, 1.52 (±0.69) at 20 dph and 2.36 (±0.9) at 27 dph. Tryptic enzyme activity level of fed larvae increased with age ($p < 0.05$) and the fluctuations of tryptic enzyme activity during the day extended with age (Figure 13 a). The daily mean (±SD) tryptic enzyme activity of fed larvae increased from 1.34 (±0.79) at 13 dph to 3.57 (±2.20) at 20 dph to 7.06 (±5.50) at 27 dph. The differences in tryptic enzyme activity between fed and unfed larvae are higher with increasing age (Figure 14). General variability in tryptic enzyme activity of fed larvae is higher with age. Tryptic enzyme activity of fed larvae (three times per day) increased with total length, with a correlation coefficient $\tau = 0.51$ (Figure 15).

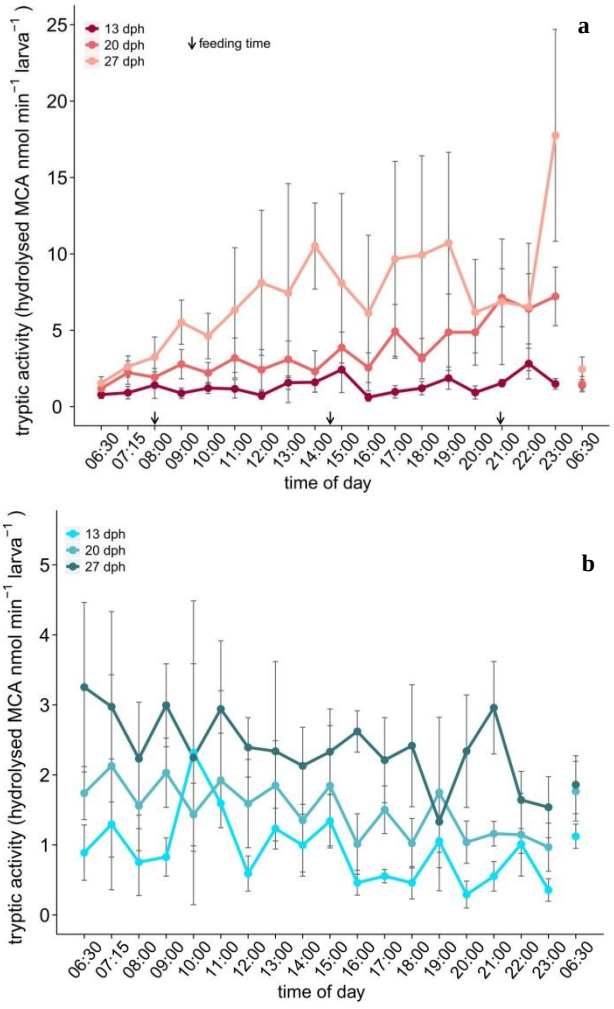

Figure 13: Tryptic enzyme activity of fed (a) and unfed (b) larvae during the diel rhythm experiment at 13 dph, 20 dph and 27 dph. Tryptic enzyme activities of fed or unfed larvae of the three trials were summarized to compare the development of the tryptic enzyme activity of fed or unfed larvae over time.

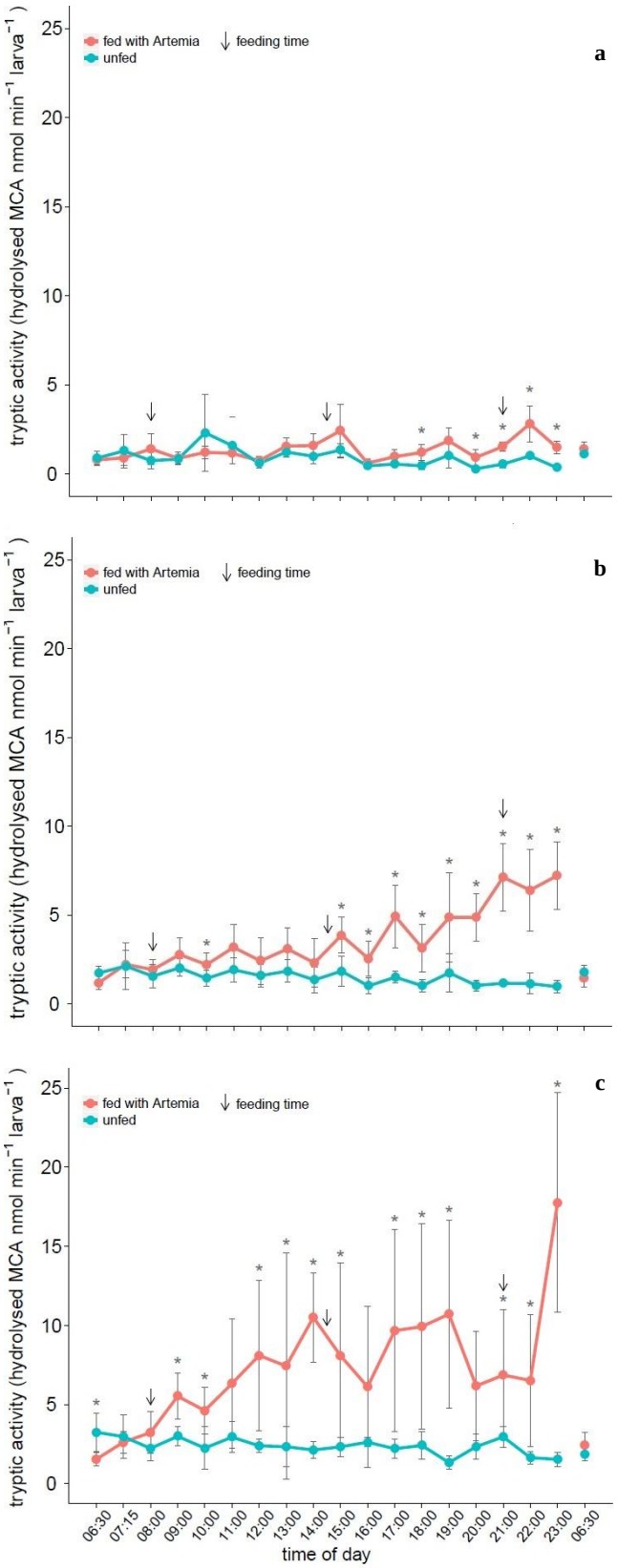

Figure 14: Tryptic enzyme activity of sea bass larvae during the diel rhythm experiment at 13 dph (a), 20 dph (b) and 27 dph (c). Y-axes were scaled at the same level to compare the diel variability of the tryptic enzyme activity between different age classes. Red lines represent larvae fed three times during the experiment. Black arrows indicate feeding time. Blue lines represent unfed larvae. Values of a, b and c are means and standard deviations of six larvae each.

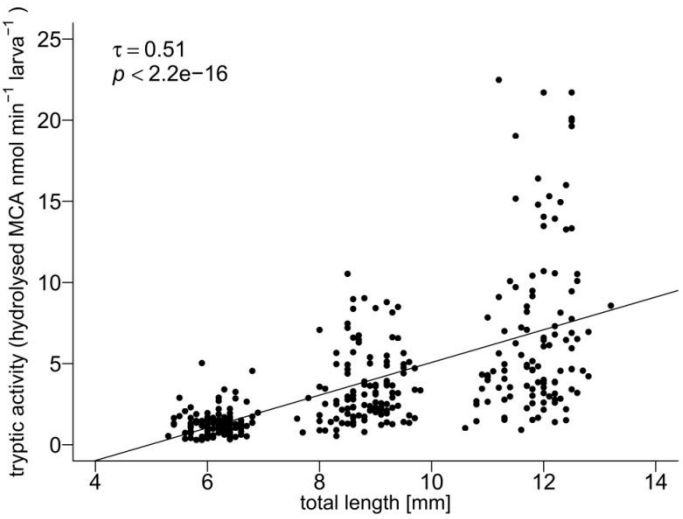

Figure 15: Correlation between tryptic enzyme activity and total length of sea bass larvae fed with *Artemia* three times during the diel rhythm experiment. Correlation coefficient $\tau = 0.51$.

3.3 Trigger experiment

Within the scope of this experiment the tryptic enzyme activity of larvae, which were either exposed to a trigger solution (ARW, BAAG, GB and MY) or only kept in sea water, were compared. For simplicity these two groups were renamed to 'trigger larvae' and 'control larvae' in the following sections.

By testing the interaction between treatment (trigger, control), sampling time (10, 20 and 30 min) and replicate (three tanks for each treatment) there were significant differences in the tryptic enzyme activity between the replicate tanks at particular times of sampling. This was the case during the AW trial at 14 dph, during the BAAG trial at 14 dph, during the GB trial at 15 dph and during all three MY trials (15 dph, 22 dph and 29 dph). Consequently in these cases differences in tryptic enzyme activity between the trigger and the control larvae were tested for each experimental phase (one control tank, one trigger tank). Figures of the single experimental phases for these cases are shown in the appendix.

3.3.1 ARW - *Artemia* rearing water

In the ARW trial at 28 dph the tryptic enzyme activity (hydrolysed MCA nmol min^{-1} $larva^{-1}$) of trigger larvae is higher than of the control larvae after 10 min and 20 min (Figure 16 c). However no statistical significant differences in tryptic enzyme activity between trigger and control larvae were found at any time of sampling (after 10, 20 and 30 min) at all three age

classes (Figure 16 a - c). By pooling tryptic enzyme activity for each treatment (trigger, control) across the three sampling times, a difference between trigger larvae and control larvae during the ARW trial at 28 dph is observed (Figure 16 d). This is confirmed by statistically significant differences in the pooled tryptic enzyme activity between trigger larvae and the control larvae during the ARW trial at 28 dph. Pooled tryptic enzyme activity increased with age (14dph, 21dph and 28dph) for both treatments ($p < 0.05$) (Figure 16 d). Tryptic enzyme activity (mean ± SD) of the trigger larvae was 0.53 (±0.31) at 14 dph and increased to 1.17 (±0.63) at 21 dph to 2.33 (±1.47) at 28 dph. For control larvae pooled tryptic enzyme activity increased from 0.60 (±0.29) at 14 dph to 1.10 (±0.55) at 21 dph to 1.80 (±0.80) at 28 dph.

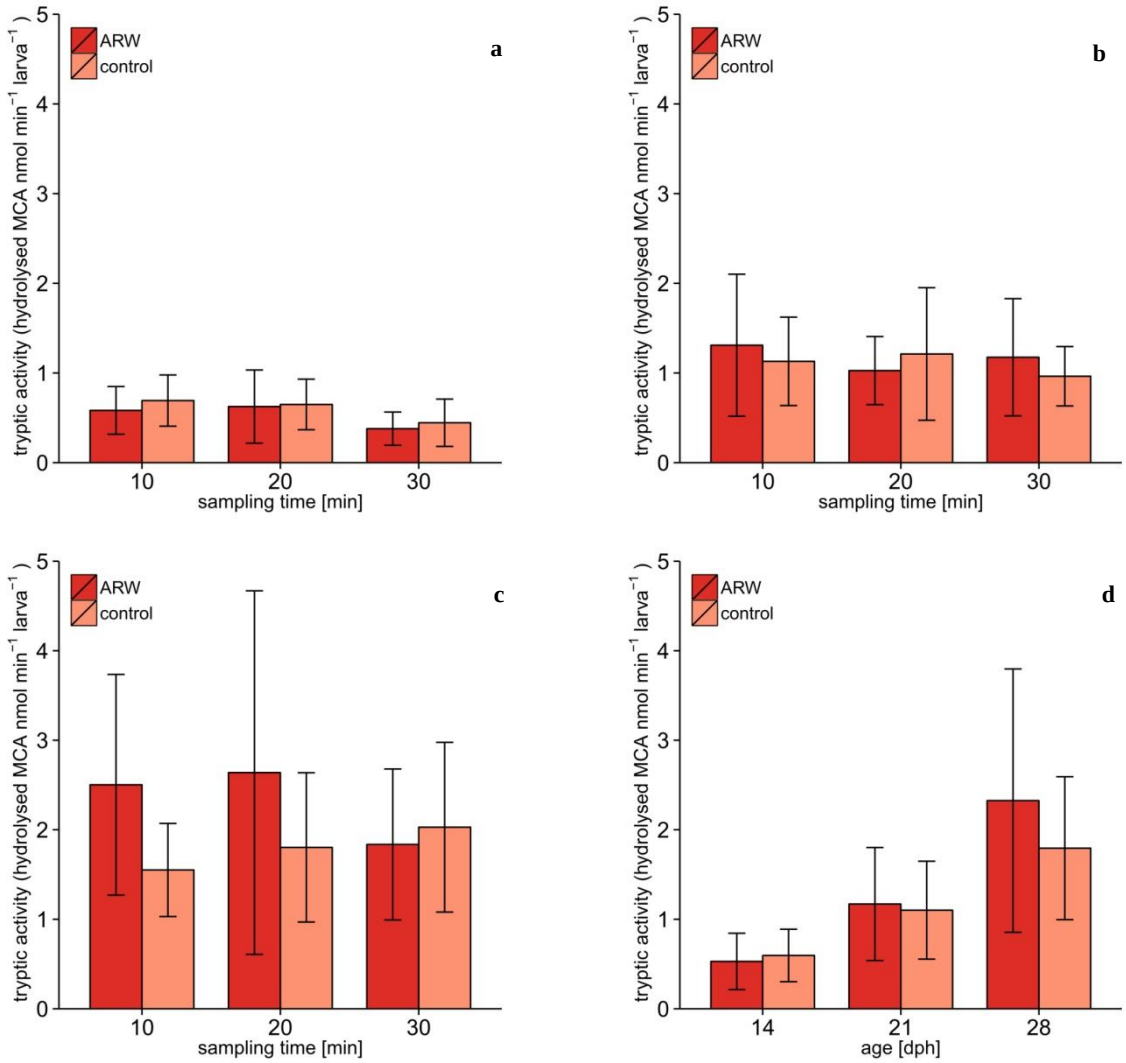

Figure 16: Tryptic enzyme activity of sea bass larvae during the trigger experiment with ARW (*Artemia* rearing water) as trigger solution at 14 dph (a), 21 dph (b), 28 dph (c) and treatment (trigger, control) pooled tryptic enzyme activities over time (d). Sampling of larvae, which were exposed to ARW, and control larvae (without any trigger) was carried out after 10, 20 and 30 min. Values (a - c) are means and standard deviations of 18 larvae from 3 replicate tanks.

3.3.2 BAAG - betaine, alanine, arginine and glycine

At all three sampling times of the BAAG trial at 28 dph the tryptic enzyme activity (hydrolysed MCA nmol min^{-1} $larva^{-1}$) of the trigger larvae is higher than of the control larvae (Figure 17 c). Further, there is a tendency of slightly higher tryptic enzyme activity of the trigger larvae during the trials at 14 dph and 20 dph than of the control larvae at almost all sampling times (Figure 17 b - c). However significant differences in tryptic enzyme activity were only found between trigger and control larvae after 10 min during the first experimental phase of the BAAG trial at 14 dph ($p < 0.05$, see appendix). Pooled tryptic enzyme activity increased with age (Figure 17 d) ($p < 0.05$). For trigger larvae tryptic enzyme activity (mean ± SD) increased from 0.76 (±0.38) at 14 dph to 1.70 (±0.88) at 21 dph to 3.23 (±1.95) at 28 dph. Pooled tryptic enzyme activity of control larvae increased from 0.66 (±0.34) at 14 dph to 1.54 (±0.84) at 21 dph to 2.63 (±1.40) at 28 dph.

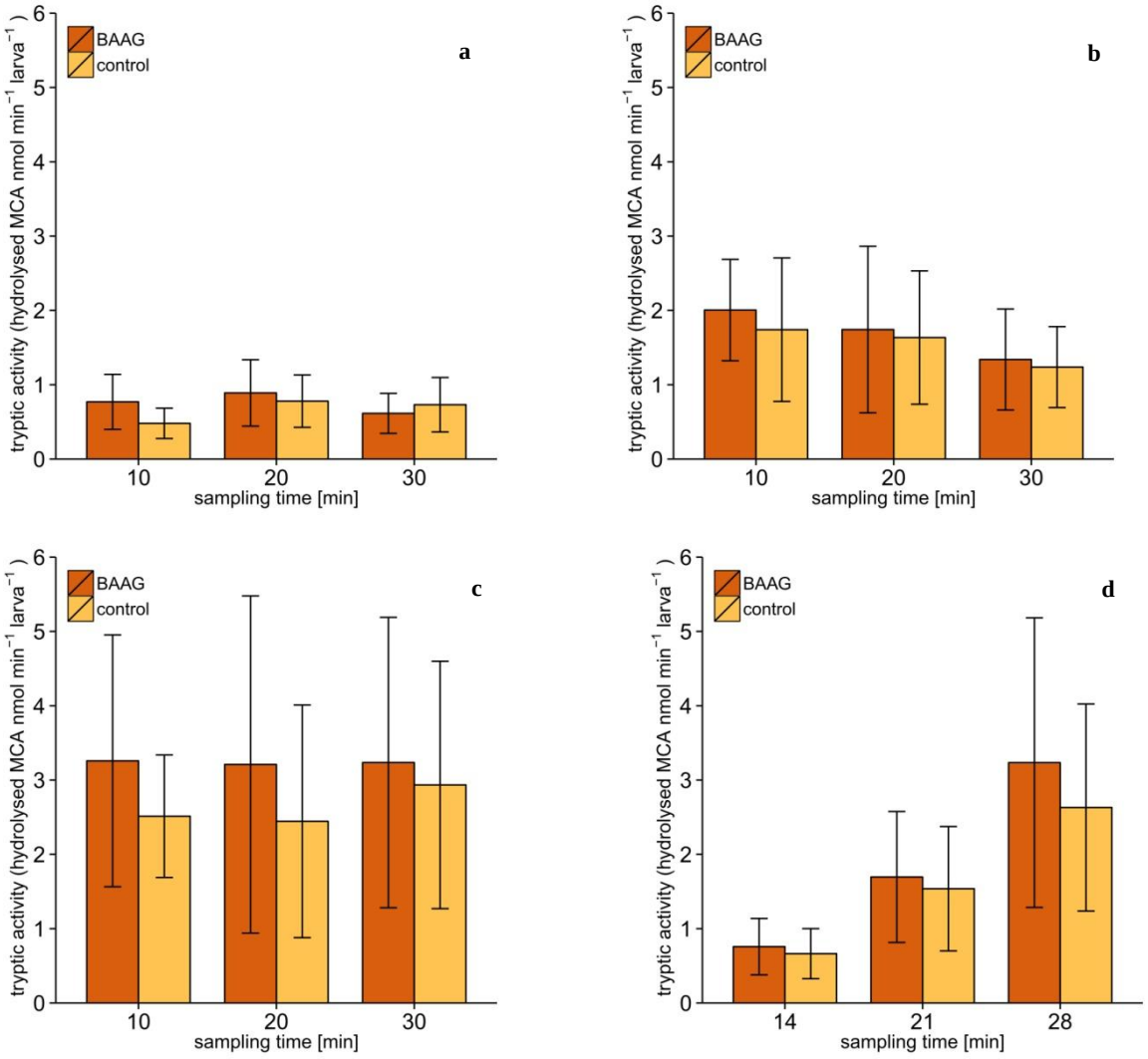

Figure 17: Tryptic enzyme activity of sea bass larvae during the trigger experiment with BAAG (mixture of betaine, alanine, arginine and glycine) as trigger solution at 14 dph (a), 21 dph (b), 28 dph (c) and treatment (trigger, control) pooled tryptic enzyme activities over time (d). Sampling of larvae, which were exposed to BAAG, and control larvae (without any trigger) was carried out after 10, 20 and 30 min. Values are means and standard deviations of 18 larvae from 3 replicate tanks.

3.3.3 GB - glycine and betaine

In the GB trials, no difference of tryptic enzyme activity (hydrolysed MCA nmol min^{-1} $larva^{-1}$) between trigger and control larvae at any time of sampling (after 10, 20 and 30 min) and at any tested age groups (15, 22, 29 and 36 dph) is observed (Figure 18 a - d). These observations were statistically confirmed ($p > 0.05$). Pooled tryptic enzyme activity increased with age (Figure 18 e) for both treatments ($p < 0.05$). At 15 dph tryptic enzyme activity (mean ± SD) was 0.54 (±0.27) and increased to 1.01 (±0.53) at 22 dph, to 2.17 (±1.21) at 29 dph and to 2.74 (±1.37) at 36 dph. For control larvae pooled tryptic enzyme activity increased from 0.55 (±0.22) at 15 dph to 0.99 (±0.51) at 22 dph, to 2.06 (±0.99) at 29 dph and to 2.77 (±1.42) at 36 dph.

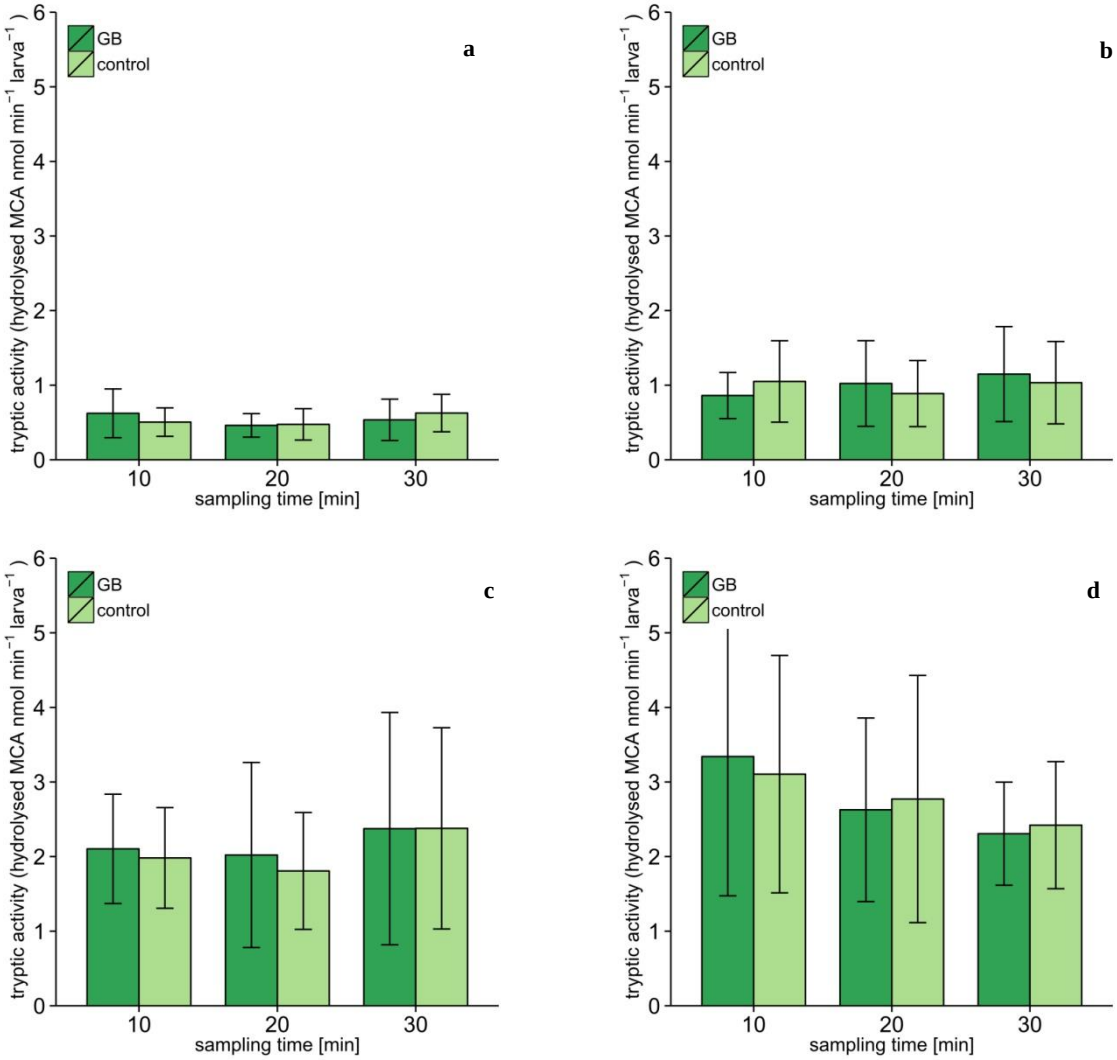

Figure 18: Tryptic enzyme activity of sea bass larvae during the trigger experiment with GB (mixture of glycine and betaine) as trigger solution at 15 dph (a), 22 dph (b), 29 dph (c), 36 dph (d) and treatment (trigger, control) pooled tryptic enzyme activities over time (e). Sampling of larvae, which were exposed to GB, and control larvae (without any trigger) took place after 10, 20 and 30 min. Values are means and standard deviations of 18 larvae from 3 replicate tanks.

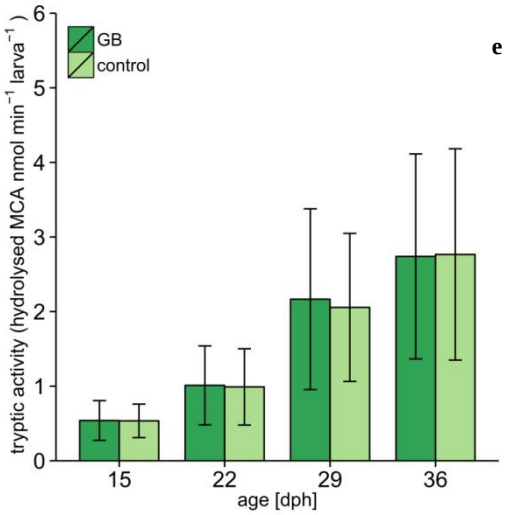

Figure 18 continued**.**

3.3.4 MY - extract of *Mytilus edulis*

At 22 dph after 10 min exposure to MY the tryptic enzyme activity (hydrolysed MCA nmol min^{-1} $larva^{-1}$) of the trigger larvae is slightly higher than of the control larvae (Figure 19 b). However, significant differences between trigger and control larvae at 22 dph after 10 min were found only during one of the three experimental phases. No differences in tryptic enzyme activity were found during the other two experimental phases at 22 dph and during the experimental phases of the other two trials at 15 dph and 29 dph (Figure 19 a - c). Pooled tryptic enzyme activity increased with age (Figure 19 d) for both, trigger and control larvae ($p < 0.05$). Tryptic enzyme activity (mean ± SD) of trigger larvae was 0.71 (±0.29) at 15 dph and increased to 1.30 (±0.77) at 22 dph and to 1.77 (±0.82) at 29 dph. Pooled tryptic enzyme activity of control larvae increased from 0.69 (±0.32) at 15 dph, to 1.28 (±0.54) at 22 dph and to 1.76 (±0.90) at 29 dph.

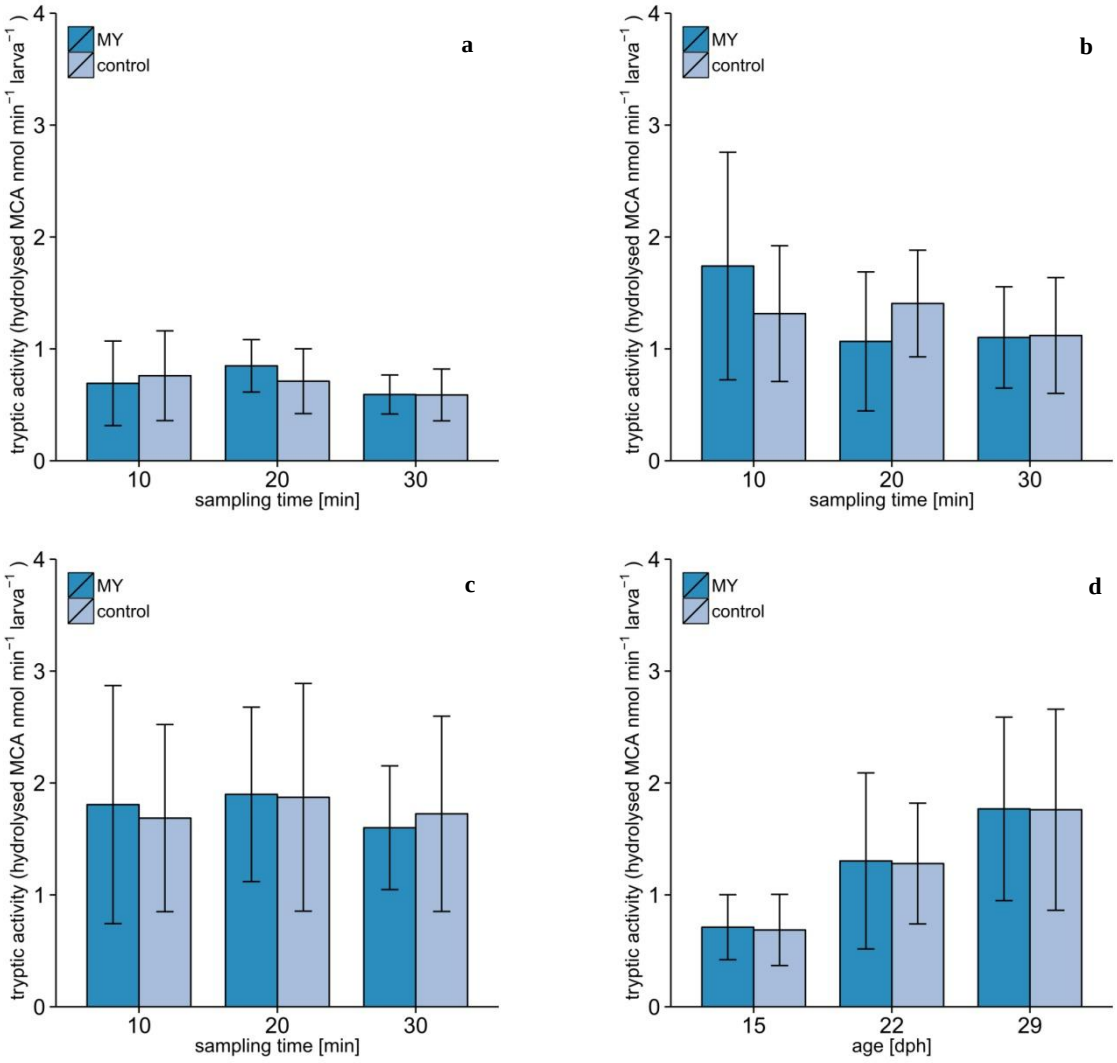

Figure 19: Tryptic enzyme activity of sea bass larvae during the trigger experiment with MY (extract of *Mytilus edulis*) as trigger solution at 15 dph (a), 22 dph (b), 29 dph (c) and treatment (trigger, control) pooled tryptic enzyme activities over time (d). Sampling of larvae, which were exposed to MY, and control larvae (without any trigger) was carried out after 10, 20 and 30 min. Values are means and standard deviations of 18 larvae from 3 replicate tanks.

4 Discussion

The study investigated the tryptic enzyme activity in European sea bass larvae at different developmental stages and attempted to:

- examine the presence of a diel rhythm in tryptic enzyme activity, and
- determine the effect of different solutions (ARW, BAAG, GB and MY) as potential triggers for the secretion of trypsinogen.

Results / methods of these experiments are discussed in the following sections.

4.1 Larval development

Success in larval development is influenced by various parameters. Abiotic factors such as temperature and light regime play an important role in yolk absorption and larval development and by careful adjustment of these factors, it is possible to maximize the proportion of yolk that is used for growth rather than utilized for activity. For Mediterranean sea bass (*Dicentrarchus labrax*) can be reported that techniques have been developed with the intention of maximizing the conversion of yolk to tissue (Brown et al., 2003). It is believed to achieve such success for instance by reducing light or rearing larvae in darkness the first days like it was performed in this study ("French method"). Another important factor influencing larval development is the feeding regime. There are several protocols with different feeding regimes using live food but also with the application of microdiets (MD). For instance Corneillie et al. (1989) reported larval survival from sea bass larvae of 22 % (until 45 dph), fed with rotifers and *Artemia*. Süzer et al. (2011) reported survival rates for sea bass larvae fed exclusively with *Artemia* (different size) of 34.1 % (until 40 dph) and additionally survival rates of 43.6 % when larvae were fed with MD from 25 dph on. In this study due to easy handling and positive experiences in larval survival and growth, sea bass larvae were exclusively fed with *Artemia* (pers. comm., B. Ueberschär). Compared with the descriptions of the different developmental stages from Russel (1976) sea bass larvae used in this study displayed normal development in terms of pigmentation, different organs (eyes, mouth, etc.) and fins during rearing period, giving the basis for running the experiments with fit larvae. The higher mortality at the onset of exogenous feeding and total absorption of yolk were possible due to providing inappropriate food in terms of quantity, accessibility or particle size (Yúfera and Darias, 2007). After 24 – 30 dph a significant amount of deformed larvae could

be observed. Deformation in larval rearing occurs when parameters such as pH, temperature, salinity or water flow are unsuitable or nutritional deficiencies or bacteria contaminate the rearing system (Barahona-Fernandes, 1982). Due to the relatively small number of deformed larvae observed in this study, this issue was not further pursued. Deformed larvae were excluded from the experiments.

4.2 Diel rhythm experiment

4.2.1 General consideration of methodological aspects

Marine fish larvae are visual feeder and therefore light is one of the most important factors responsible for diel variability in feeding behavior (Blaxter, 1986; Hunter, 1980). Based on this, the sampling to examine the diel variability in tryptic enzyme activity of unfed and fed larvae was performed at specific times of the day and night. A more frequent (hourly) sampling enabled greater precision in monitoring possible changes in tryptic enzyme activity. To depict more general pattern for example a circadian rhythm, a frequent sampling during night is necessary. However, the first samplings which were taken before sunrise may be a first indication. Partially filled guts of larvae sampled before sunrise may indicate that some larvae seem to feed during night. Nighttime (darkness) lasted 8 hours so it seems to be unlikely that the larval guts were still filled from last feeding. People Le Ruyet et al. (1993) reported a gut evacuation time of approximately 2.0 - 4.5 hours for sea bass larvae fed with *Artemia*.

4.2.2 Diel rhythm of tryptic enzyme activity

Fed larvae showed diel fluctuations in tryptic enzyme activity in dependence of gut fullness at all tested age classes. Tryptic enzyme activities tended to be increasing during day. The associated fluctuations in tryptic enzyme activity and gut fullness allow conclusions to be drawn on variations in the feeding activity during the day. A similar pattern in diel variations in tryptic enzyme activity, when larvae were fed with live food at least two times during the day, has previously been observed (Tillner et al., 2014; Ueberschär, 1995). At the beginning of each trial, tryptic enzyme activity of fed larvae increased prior to first feeding (06:30 – 08:00). This increase could be explained by light-induced feeding on *Artemia*, which remained in the tanks from the previous evening with the last feeding event. It was demonstrated, that larvae could store large quantities of trypsinogen in the pancreas in periods

deprived of food and could quickly response with secretion of trypsinogen upon a feeding event (Ueberschär et al., 1992).

In **13 dph** - old larvae, a trend to increasing tryptic activities occurred as a response to the second and last feeding. Similar feeding induced increases in tryptic enzyme activity were reported from Pedersen and Hjelmeland (1988) and Ueberschär (1993). Noticeably is the marked decrease in tryptic enzyme activity of fed larvae compared to the activity level of unfed larvae, for instance at 16:00. Tillner et al. (2013), who noticed similar pattern after feeding cod larvae with rotifers, assumed that the obvious decrease might be a result of immediate binding of trypsin on food particles, because it is presumed that bound trypsin cannot be measured with a tryptic activity assay (Ueberschär, 2006).

In **20 dph** - old larvae**,** following the first two feeding events, the tryptic enzyme activity was increasing continuously. However, the third feeding in the evening seems to have no further effect on tryptic enzyme activity of fed larvae. The increase in tryptic enzyme activity is correlated to a continuous increase in gut fullness, indicating that larvae fed continuously and feeding activity increased during the day. No further effect on tryptic enzyme activity after the third feeding, although larvae had full guts, indicates that larvae reached the limit of their tryptic enzyme capacity. In aquaculture larvae are reared under conditions different from conditions found in their natural environment, for instance long lightning periods or even 24 h illumination. These changes of natural conditions are intended to increase larval feeding activity and therefore larval growth, because larvae feed continuously due to long lighting periods. However, it should be considered that larvae, particularly early larval stages, might not be capable, like properly in this case, to exploit the proposed feeding regime due to natural limited digestive capacity.

In **27 dph** - old larvae, a feeding induced increase in tryptic enzyme activity of fed larvae was observed after the first and third feeding. A stagnation and general decline in tryptic enzyme activity was observed between the time of second and third feeding. If we consider the associated gut fullness, this stagnation / decrease in tryptic enzyme activity seems to be caused by a minor feeding activity. Until 25 dph larvae were fed with instar I nauplii. At 26 / 27 dph larvae were fed with a mixture of instar I and enriched instar II nauplii. However, due to the significant positive response in feeding activity (tryptic activity / gut fullness) after first and third feeding it seems to be unlikely that the food change caused the observed periods of minor feeding activities. It could be that larvae at this developmental stage change their feeding pattern over the day to higher activities for the feeding in the morning and evening

and lower feeding activities during afternoon due to satisfied feeding needs at this time. But to confirm this vague assumption further investigation with larger samples sizes is needed.

Tryptic enzyme activity of **unfed larvae** slightly decreased in general during the day at all age classes. Individual higher values in tryptic enzyme activity during the first hours of the day are associated with partially filled guts. Pedersen and Hjelmeland (1988) and Pedersen and Andersen (1992) reported that larvae retain trypsin in the gut for several hours after a meal. The slight decrease in tryptic enzyme activity of the unfed larvae during the day might be related with the gradually re-absorption of retained trypsin or related to the binding of retained trypsin to the gut epithelium (Pedersen and Hjelmeland, 1988).

At **13 dph** the tryptic enzyme activity of unfed larvae showed on a lower tryptic activity level similar fluctuation patterns at the time of second and third feeding like fed larvae. Such a synchronization of tryptic enzyme activity to planned feeding events of unfed larvae has already been described for sea bass larvae by Tillner et al. (2014). However, in unfed larvae at **20 dph** and **27 dph** this pattern in tryptic enzyme activity was not observed. At these age classes the tryptic enzyme activity of unfed larvae was reasonable steady compared to the fed larvae, like it was observed in other studies for starved larvae (Fujii et al., 2007; García-Ortega et al., 2000; Ueberschär, 1995).

4.2.3 Comparison between age classes

Positive correlations of tryptic enzyme activity with larval size and developmental stage (age) correspond to results of previous studies. For instance, tryptic enzyme activity of fed larvae has been observed to increase with larval size in herring (*Clupea harengus*), haddock (*Melanogrammus aeglifinus*) and cod (*Gadus morhua*) larvae (MacKenzie et al., 1999; Tillner et al., 2013; Ueberschär, 1993). Additionally, the increase with age in tryptic enzyme activity level of fed larvae and short-term unfed larvae (one day deprived of food) was previously shown by Ueberschär (1995) for fed and short-term starved (three days deprived of food) herring larvae.

Differences in tryptic enzyme activity between unfed and fed larvae seem to increase with larval age. This observation could be explained by the increasing gut lumen and increasing tryptic activity capacity during larval development (Ueberschär, 2006).Therefore fed larvae are able to ingest more prey organisms due to a greater gut lumen and could response with higher trypsinogen secretion due to a higher tryptic capacity. This also explains the increasing

correlation coefficient between gut fullness and tryptic enzyme activity with increasing age. Tryptic enzyme activity of unfed larvae remains at a basis level due to absence of prey organisms.

4.2.4 Summary of diel rhythm experiment

The diel rhythm experiment revealed that unfed and fed larvae had different patterns in tryptic enzyme activity during the day. In terms of the (I) question, if enzyme activity level changes during the day, it can be concluded that fed larvae showed feeding induced diel variations in tryptic enzyme activity. Unfed larvae seem to maintain a reasonable steady baseline level in tryptic enzyme activity during the day (at least 20 and 27 dph) compared to fed larvae. For the (II) question, if there is a change in the diel rhythm of tryptic enzyme activity with age, it can be concluded that the diel rhythm of tryptic enzyme activity differ between the age classes for fed larvae due to different feeding activities during the day and different tryptic activity capacity. Younger larvae (13 dph) with a limited tryptic activity capacity show increasing tryptic activities, depending on their feeding activity, mainly to feedings later on the day. In older larvae (20 dph), tryptic activity is in general correlated with continuously increasing feeding activities during the day, but has limitations with a third meal in the evening, where tryptic activity seems not to increase. In 27 dph – old larvae, increasing tryptic enzyme activity seems also be related to increasing feeding activity, but feeding activity seems momentary to stagnate or even decrease after second feeding. The baseline level of tryptic enzyme activity for larvae deprived of food for several hours was slightly higher with increasing age.

4.3 Trigger experiment

4.3.1 General consideration of methodological aspects

The different trials were performed with best care and attention, however it could not be entirely ensured that handling of larvae during the trials might have had an effect on the performance of the larvae.

Other issues, which might have an influence or have to be considered by analyzing the results are discussed in the following paragraphs.

Global test statistics revealed significant differences in tryptic enzyme activity of fish larvae between the three replicate tanks (trigger and control) in some trials of the trigger experiment. For instance, differences between replicate tanks were found for all trials at the first age class. Most likely this was caused by the separation of the trial into three consecutive experimental phases. One experimental phase took 30 min, so the entire trial took at least 2 h. Considering the results of the diel rhythm experiment at 13 dph the tryptic enzyme activity of unfed larvae could fluctuated considerably over a time period of 2 - 2.5 h, compared with the general low tryptic activity level at this age. The experimental phases were evaluated individually if differences between the replicate tanks were found. For further studies it is recommended to start the experimental phases at the same time to reduce the problem of overlaying diel fluctuations in tryptic enzyme activity between the experimental phases.

Based on the naturally occurring developmental differences among conspecifics even in the same larval cohort, the individual tryptic activity capacity of larvae could differ considerably. This has to be considered by interpreting the results of the experiment. Large variations (standard deviation) may camouflage differences, particularly at early larval stages, in which in general tryptic activity levels are very low. The tryptic enzyme activity increased with larval size therefore samples of several larvae, which differ immensely in length could distort the results (mean values). To reduce this effect, the tryptic enzyme activity results were calculated per larvae and normalized to one mm of larval length. By comparing the results of these two calculations no considerable differences were found. Consequently, it was valid to calculate the tryptic enzyme activity per larva.

Another problem, which has to be considered by interpreting the results is the performance of the four trigger trials at different times of the day. Larvae for the trigger experiment were deprived of food from beginning of the experimental day to ensure larvae have empty guts to the start of the different trails. The diel rhythm experiment demonstrated that unfed larvae maintain a reasonable steady basis level in tryptic enzyme activity during the day (at least at 20 dph and 27 dph). Therefore, it was believed that larvae of the different trials should have similar tryptic activity basis levels. However, statistical tests revealed several differences in tryptic enzyme activity level between control larvae from the four different trials. Thus, the basis level in tryptic enzyme activity of larvae from the four trails was different. These differences do not cause problems for the comparison of the tryptic enzyme activity between control and trigger larvae of the individual trials, but for the comparison between different

trials. Accordingly a statistical comparison between different trials of one age class was omitted.

4.3.2 Effect of *Artemia* rearing water (ARW)

ARW treatment triggered an enhanced trypsinogen secretion in sea bass larvae at 28 dph after 10 and 20 min. Statistics did just confirm a significant difference for the comparison of the treatment pooled data not for the individual comparisons at each sampling time due to high standard deviations and probably the small sample size. No effect of ARW on tryptic enzyme activity in larvae at 14 and 20 dph were observed.

Generally, it is known that plankton organisms release metabolites, which are considered to act as attractant for fish larvae. Additionally, Kolkovski et al. (1997a) reported that *Artemia* rearing water triggers feeding rates in 20 - days - old sea bream larvae. However observations on the larvae, which were exposed to ARW for 30 minutes, revealed no significant effect in tryptic enzyme activity. This missing response in tryptic enzyme activity after 30 min exposure might be explained by gradually re-absorption of trypsin over the time due to the absence of any substrate. Pedersen and Hjelmeland (1988) reported such mechanism as a result of the absence of further ingestion of prey organisms or food particles.

The tested concentration of ARW might have been too low to trigger the maximum trypsinogen secretion of larvae at 28 dph. This hypothesis is based on the observation that the tryptic enzyme activity of the trigger larvae seem to be lower than the possible tryptic capacity of sea bass larvae at this age class, if we consider the tryptic enzyme activity level of fed larvae (diel rhythm experiment) as an approximate value of the tryptic activity capacity.

Absence of any effect of ARW during the trials at 14 and 21 dph might be related to the developmental state of the sensory organs. It is known that olfactory and gustatory sensory cells are differentiated very early and olfaction plays an active role in detection of prey organism for most of the larvae (Knutsen, 1992; Rønnestad et al., 2013). However, it is believed that further differentiation of organs, being able to detect chemical stimuli, takes place later in larval development (Rønnestad et al., 2013). Consequently, chemical stimulus alone might be insufficient to trigger the secretion of trypsinogen at this early larval stage. However, Webb and Holt (2008) reported an increase of tryptic enzyme activity of red drum larvae at 6 dph only due to the chemical stimuli of soluble components of rotifers. Additionally, Knutsen (1992) observed a positive effect on behavior patterns related to

feeding in North Sea turbot larvae and Dover sole larvae at 4-5 and 9 dph, which were exposed only to chemical stimuli. Based on these findings, it seems to be more likely that even the youngest larval stage can be stimulated by an individual chemical. However, for most species the developmental state of sensory organs at different larval stages is unknown (Rønnestad et al., 2013).

4.3.3 Effect of betaine, alanine, arginine and glycine (BAAG)

Larvae at 28 dph respond with an increase in trypsinogen secretion to the exposure of BAAG, particularly after 10 and 20 min exposure. Statistics however, did not confirm significant differences ($p < 0.05$) due to high standard deviations and probably the small sample size. During the trails at 14 and 20 dph no clear response in higher trypsinogen secretion could be observed, however in most of the samples the tryptic activity of larvae exposed to the trigger are slightly higher.

Although not significant in statistical tests, it is supposed that the results nevertheless demonstrate that BAAG has to be considered as potential trigger for trypsinogen secretion in larvae at 28 dph. Betaine, alanine, arginine and glycine are known to be found in prey organism and released from prey organism, thus being probably an important fed attractant (Hamre et al., 2013; Kolkovski et al., 1997a). For instance Kolkovski et al. (1997a) attributed higher feeding rates in sea bream larvae, which were exposed to *Artemia* rearing water, to the presence of these four metabolites. According to the results from Kolkovski the tested BAAG concentrations were adapted to the concentrations found in *Artemia* rearing medium (Kolkovski et al., 1997a). The reduced trypsinogen secretion after 30 min exposure to BAAG might again be explained by gradually reabsorption of trypsin over the time due to the absence of any substrate (Pedersen and Hjelmeland, 1988). Meaning ARW as chemical stimuli induces secretion of an initial amount of trypsinogen into the gut lumen, however any further release of trypsinogen depends on a mechanical trigger, which means food organisms or food particles need to pass the gut.

The findings of the trials with younger larvae (14 dph and 21 dph) might indicate a stimulation of the trypsinogen secretion, if we consider that in general early larval stages have a low tryptic activity capacity and a higher response in trypsinogen secretion might not be possible. However, considering the results from the diel rhythm experiment at 13 dph it could be observed that the trigger larvae respond with a tryptic activity level far below the tryptic

activity capacity. Therefore it seems to be unlikely that the observed slightly higher tryptic activity levels of trigger larvae indicate considerable differences in the tryptic enzyme activity between trigger and control larvae at this age class.

4.3.4 Effect of glycine and betaine (GB)

GB did not trigger trypsinogen secretions of sea bass larvae at all tested age classes (15 – 36 dph). These findings are unexpected, if considering for example results from Knutsen (1992), who demonstrated a positive effect of glycine and betaine on feeding behavior pattern (swimming, snapping and darting) in North Sea turbot and Dover sole larvae (4-5 dph and 9 dph), and a recent study with sea bass larvae observed enhanced swimming activity, when larvae were exposed to a mixture of glycine and betaine (Sommerfeld, 2014). It has to be noted, however, that most of the experiments from Sommerfeld (2014) were conducted with older sea bass larvae (37 – 65 dph).

The tested GB concentration was between concentrations of Knutsen (1992) and Sommerfeld (2014) and was in a range of amino acid concentrations found to have a positive effect on feeding behavior (Kolkovski, 2008). This indicates that the tested concentration was generally adequate to be detected by fish larvae. However, if we consider that the response to different amino acids could differ between species like it was observed from Knutsen (1992), than it might be also possible that the response to different concentrations of amino acids could differ between species and age classes. If we consider this suggestion, than it might be possible that the tested concentration was inadequate after all, because Sommerfeld (2014) reported a response of sea bass larvae at concentrations four times higher than the tested GB concentration of this study.

4.3.5 Effect of *Mytilus edulis* extract (MY)

In one of three experimental phases, larvae at 22 dph seem to respond with an increase in trypsinogen secretion after 10 min exposure of MY. However, due to the absence of any further effect of MY on the trypsinogen secretion at this age group and also during the trials at the other two age classes, it becomes clear that MY has no effect on the trypsinogen secretion.

Although several studies reported that synthetic or natural hydrolysates / extracts of marine organism positively affect feeding behavior in fish larvae (Kolkovski and Tandler, 2000;

Kolkovski, 2008; Kolkovski et al., 2000), *Mytilus* extract has never been found to be positively affecting feeding behavior or trypsinogen secretion in fish larvae. Positive impacts have only been found in juvenile and adult fish (Carr et al., 1996; Tandler et al., 1982). In general hydrolysates / extracts are known to act as fed attractant, because they contain digested protein components like free amino acids. *Mytilus edulis* contains many different amino acids. After Tandler et al. (1982) and Carr et al. (1996) *Mytilus edulis* contain particular high concentrations of alanine, glycine and glumatic acid. Considering that during the other trials some of these amino acids have already shown their potential to trigger the trypsinogen secretion in sea bass larvae, the tested concentration of MY might be inadequate to trigger the trypsinogen secretion. This suggestion can be confirmed by the fact that generally the quantification of the concentration of extracts is much more difficult compared to mixture of amino acids (Kolkovski, 2008) and Zandee et al. (1980) observed in *Mytilus edulis* clear annual variations in the biochemical composition (e.g. quantity of amino acids).

4.3.6 Summary of the trigger experiments

The trigger experiment revealed different results for the four tested trigger solutions. In terms of the (I) question, if larvae exposed to different trigger solutions (ARW, BAAG, GB, MY) have higher tryptic enzyme activities than control larvae, it can be concluded that some substances (mixtures), such as ARW and BAAG, have the potential to trigger the secretion of trypsinogen. However, it cannot be excluded that the other tested substances (GB and MY) also are potential trigger for the secretion of trypsinogen, for instance at a different concentration level. For the (II) question, if the effect of the trigger solutions on tryptic enzyme activity changes with age, it can be concluded that the effect changes with age in the way that a (clear) response of the secretion of trypsinogen to a chemical stimuli alone might be not possible for sea bass larvae until a certain developmental stage. For the early larval stages an additional trigger such as mechanical stimuli due to food ingestion might be necessary to trigger the secretion of trypsinogen. Hjelmeland et al. (1988) have already reported a slightly higher trypsin secretion of herring larvae, which ingest polystyrene spheres compared to non-feeding larvae and compared this effect with the neural regulation of pancreatic function in mammals stimulated by the distension of the stomach.

4. 4 Conclusion and outlook

Enhanced knowledge about feeding behavior and digestive physiology is a key target to enable further improves larval rearing protocols. For this reason studies to improve larval rearing protocols such as the application of MD, but also further studies to advance the knowledge about the ontogenetic changes and specifically digestive physiology during larval development are necessary.

To learn more about the digestive physiology of larvae and also to evaluate the observations of previous studies, experiments to examine the diel rhythm of tryptic enzyme activity in sea bass larvae were performed. For these experiments it can be concluded that feeding-induced diel variations in tryptic enzyme activity can be observed in fed sea bass larvae. Unfed larvae seem to maintain a reasonable steady basis level in tryptic enzyme activity during the day. Knowledge about diel variations in tryptic enzyme activity and feeding activity could help to adjust feeding schedules to possible observed higher activities during the day.

The main focus of this thesis was to find substances, which stimulate the secretion of trypsinogen of sea bass larvae, and consequently, facilitate the increase in tryptic enzyme activity. For these experiments it can be concluded that *Artemia* rearing water or at least components of this medium such as specific amino acids (betaine, alanine, arginine and glycine) are potential substances to trigger the trypsinogen secretion of sea bass larvae. This response in the secretion of trypsinogen could be associated with the unconditional response described by Pavlov to unconditional stimuli such as BAAG and ARW. Further experiments with these substances might be useful to examine the adequate concentration to stimulate the highest possible tryptic activity capacity at different larval stages. Although the solution of glycine and betaine and the extract of *Mytilus edulis* failed to trigger a positive response in terms of increased trypsinogen secretion, these substances should not be neglected as potential trigger. Further investigations to test the effect of different concentrations of these substances on the trypsinogen secretion might be useful.

To go one step further, studies which test the response of larvae (feeding rate, growth rates, etc.) to the supplementation of substances such as BAAG in MD, should be performed. Additionally, the question if and from which developmental stage larvae of different species respond clearly to a chemical stimuli without any further stimuli such as food ingestion (mechanical stimulation) or the presence of prey organism (visual) should be considered. All these observations help to advance the development of a highly efficient MD and therefore might help in the future to fill some gaps / mismatches in larval rearing protocols.

5 References

Barahona-Fernandes, M.H., 1982. Body deformation in hatchery reared European sea bass *Dicentrarchus labrax* (L). Types, prevalence and effect on fish survival. J. Fish Biol. 21, 239–249.

Baskerville-Bridges, B., Kling, L.J., 2000. Early weaning of Atlantic cod (*Gadhus morhua*) larvae onto a microparticualte diet. Aquaculture 189, 109–117.

Ben Khemis, I., Audet, C., Fournier, R., de la Noüe, J., 2003. Early weaning of winter flounder (*Pseudopleuronectes americanus* Walbaum) larvae on a commercial microencapsulated diet. Aquac. Res. 34, 445–452.

Bengston, D.A., Lydon, L., Ainley, J.D., 1999. Green-water rearing and delayed weaning improve growth and survival of summer flounder. N. Am. J. Aquac. 61, 239–242.

Blaxter, J.H.S., 1986. Development of sense organs and behaviour of teleost larvae with special reference to feeding and predator avoidance. Trans. Am. Fish. Soc. 115, 98–114.

Brown, J.A., Minkoff, G., Puvanendran, V., 2003. Larviculture of Atlantic cod (*Gadus morhua*): progress, protocols and problems. Aquaculture 227, 357–372.

Cahu, C.L., Zambonino Infante, J.L., Péres, A., Quazuguel, P., Le Gall, M.M., 1998. Algal addition in sea bass (*Dicentrarchus labrax*) larvae rearing: Effect on digestive enzymes. Aquaculture 161, 479–489.

Carr, W.E.S., Netherton, J.C., Gleeson, R.A., Derby, C.D., 1996. Stimulants of feeding behavior in fish: Analyses of tissues of diverse marine organisms. Biol. Bull. 190, 149–160.

Conceição, L.E.C., Yúfera, M., Makridis, P., Morais, S., Dinis, M.T., 2010. Live feeds for early stages of fish rearing. Aquac. Res. 41, 613–640.

Corneillie, S., Noterdaeme, L., Ollevier, F., 1989. Culture of sea bass larvae (*Dicentrarchus labrax*) in completely closed recirculation systems with artificial seawater. Aquac. - A Biotechnolgy Prog.

Dabrowski, K., 1984. The feeding of fish larvae: present state of the art and perspectives. Reprod. Nutr. Dev. 24, 807–833.

Engrola, S., Figueira, L., Conceição, L.E.C., Gavaia, P.J., Ribeiro, L., Dinis, M.T., 2009. Co-feeding in *Senegalese sole* larvae with inert diet from mouth opening promotes growth at weaning. Aquaculture 288, 264–272.

FAO, 2014. The State of World Fisheries and Aquaculture 2014. Rome.

Fujii, A., Kurokawa, Y., Kawai, S., Yoseda, K., Dan, S., Kai, A., Tanaka, M., 2007. Diurnal variation of tryptic activity in larval stage and development of proteolytic enzyme activities of malabar grouper (*Epinephelus malabaricus*) after hatching. Aquaculture 270, 68–76.

García-Ortega, A., Verreth, J., Segner, H., 2000. Post-prandial protease activity in the digestive tract of African catfish *Clarias gariepinus* larvae fed decapsulated cysts of *Artemia*. Fish Physiol. Biochem. 22, 237–244.

Goh, Y., Tamura, T., 1980. Effect of amino acids on the feeding behaviour in red sea bream. Comp. Biochem. Physiol. 66C, 225–229.

Govoni, J.J., Boehlert, G.W., Watanabe, Y., 1986. The physiology of fish digestion in fish larvae. Environ. Biol. Fishes 16, 59–77.

Hamre, K., Yúfera, M., Rønnestad, I., Boglione, C., Conceição, L.E.C., Izquierdo, M., 2013. Fish larval nutrition and feed formulation: Knowledge gaps and bottlenecks for advances in larval rearing. Rev. Aquac. 5.

Hjelmeland, K., Pedersen, B.H., Nilssen, E.M., 1988. Trypsin content in intestines of herring larvae, *Clupea harengus*, ingesting inert polystyrene spheres or live crustacea prey. Mar. Biol. 98, 331–335.

Hunter, J.R., 1980. The feeding behavior and ecology of marine fish larvae, in: Fish Behavior and Its Use in the Capture and Culture of Fishes. Vol.5.. 287–330.

Izquierdo, M.S., 1996. Essential fatty acid requirements of cultured marine fish larvae. Aquac. Nutr. 2, 183–191.

Kamisaka, Y., Rønnestad, I., 2011. Reconstructed 3D models of digestive organs of developing Atlantic cod (*Gadus morhua*) larvae. Mar. Biol. 158, 233–243.

Kanazawa, A., Koshio, S., Shin-Ichi, T., 1989. Growth and survival of larval red sea bream *Pagrus major* and Japanese founder *Paralichthyus olivaceus* fed microbound diets. J. World Aquac. Soc. 20, 31–37.

Kjorsvik, E., Pittman, K., Pavlov, D., 2004. Chapter 6. From fertilisation to the end of metamorphosis - functional development, in: Culture of Cold - Water Marine Fish. 204–278.

Knutsen, J., 1992. Feeding behavior of North Sea turbot (*Scophthalmus Maximus*) and Dover sole (*Solea solea*) larvae elicited by chemical stimuli. Mar. Biol. 113, 543–548.

Kolkovski, S., 2008. Advances in marine fish larvae diets, in: Cruz Suárez, L.E., Ricque Marie, D., Tapia Salazar, M., Nieto López, M. G. Villarreal Cavazos, D.A. Lazo Corvea, J.P. Y Viana, M.T. Editores. 2008. Avances En Nutrición Acuícola IX. Memorias Del Noveno Simposium Internacional de Nutrición Acuícola. 24 - 2. 20–45.

Kolkovski, S., Arieli, A., Tandler, A., 1997a. Visual and chemical cues stimulate microdiet ingestion in sea bream larvae. Aquac. Int. 5, 527–536.

Kolkovski, S., Czesny, S., Dabrowski, K., 2000. Use of Krill hydrolysate as a feed attractant for fish larvae and juveniles. J. World Aquac. Soc. 31, 81–88.

Kolkovski, S., Koven, W., Tandler, A., 1997b. The mode of action of *Artemia* in enhancing utilization of microdiet by gilthead seabream *Sparus aurata* larvae. Aquaculture 155, 193–205.

Kolkovski, S., Tandler, A., 2000. The use of squid protein hydrolysate as a protein source in microdiets for gilthead seabream *Sparus aurata* larvae. Aquac. Nutr. 6, 11–15.

Koven, W., Rojas-Garcia, C.R., Finn, R.N., Tandler, A., Rønnestad, I., 2002. Stimulatory effect of ingested protein and/or free amino acids on the secretion of the gastro-endocrine hormone cholecystokinin and on tryptic activity, in early-feeding herring larvae, *Clupea harengus*. Mar. Biol. 140, 1241–1247.

Lazo, J.P., Holt, G.J., Arnold, C.R., 2000. Ontogeny of pancreatic enzymes in larval red drum *Sciaenops ocellatus*. Aquac. Nutr. 6, 183–192.

Light, A., Janska, H., 1989. Enterokinase (enteropeptidase): comparative aspects. Trends Biochem. Sci. 14, 110–112.

MacKenzie, B.R., Ueberschär, B., Basford, D., Heath, M., Gallego, A., 1999. Diel variability of feeding activity in haddock (*Melanogrammus aeglifinus*) larvae in the East Shetland area, North Sea. Mar. Biol. 135, 361–368.

Mackie, a. M., Adron, J.W., Grant, P.T., 1980. Chemical nature of feeding stimulants for the juvenile Dover sole, *Solea solea* (L.). J. Fish Biol. 16, 701–708.

Moretti, A., Fernadez-Criado, M.P., Cittolin, G., Guidastri, R., 1999. Manual on hatchery production of sea sass and gilthead seabream. FAO 1, 194.

Pedersen, B.H., Andersen, K.P., 1992. Induction of trypsinogen secretion in herring larvae (*Clupea harengus*). Mar. Biol. 112, 559–565.

Pedersen, B.H., Hjelmeland, K., 1988. Fate of trypsin and assimilation efficiency in larval herring (*Clupea harengus*) following digestion of copepods. Mar. Biol. 97, 467–476.

Pedro Cañavate, J., Fernández-Díaz, C., 1999. Influence of co-feeding larvae with live and inert diets on weaning the sole *Solea senegalensis* onto commercial dry feeds. Aquaculture 174, 255–263.

People Le Ruyet, J., Alexandre, J.C., Thébaud, L., Mugnier, C., 1993. Marine fish larvae feeding: formulated diets or live prey? J. World Aquac. Soc. 24, 211–224.

Ribeiro, L., Zambonino-Infante, J.L., Cahu, C., Dinis, M.T., 1999. Development of digestive enzymes in larvae of *Solea senegalensis*, Kaup 1858. Aquaculture 179, 465–473.

Rønnestad, I., 2002. Control and efficiency of digestive function of marine fish larvae. Av. en Nutr. Acuícole VI. Memorias del VI Simp. Int. Nutr. Acuícola 152–165.

Rønnestad, I., Thorsen, A., Finn, R.N., 1999. Fish larval nutrition: A review of recent advances in the roles of amino acids. Aquaculture 177, 201–216.

Rønnestad, I., Yúfera, M., Ueberschär, B., Ribeiro, L., Sæle, Ø., Boglione, C., 2013. Feeding behaviour and digestive physiology in larval fish: Current knowledge, and gaps and bottlenecks in research. Rev. Aquac. 5.

Rosenlund, G., Stoss, J., Talbot, C., 1997. Co-feeding marine fish larvae with inert and live diets. Aquaculture 155, 183–191.

Russel, F.S., 1976. The eggs and planktonic stages of british marine fishes.

STECF, 2013. The Economic Performance of the EU Aquaculture Sector (STECF 13-29).

Sommerfeld, D., 2014. Dissolved amino acids and natural extracts as attractant for sea bass larvae (*Dicentrarchus labrax* Linneaus, 1758).

Süzer, C., Kamaci, O.H., Deniz, C., Saka, Ş., Firat, K., Karacaoglan, A., 2011. Early weaning of sea bass (*D. labrax*) larvae: effects on growth performance and digestive enzyme activities. Turkish J. Fish. Aquat. Sci. 11, 491–497.

Suzer, C., Saka, Ş., Firat, K., 2006. Effects of illumination on early life development and digestive enzyme activities in common pandora *Pagellus erythrinus* L. larvae. Aquaculture 260, 86–93.

Tandler, A., Berg, B.A., Kissil, G.W., Mackie, A.M., 1982. Effect of food attractants on appetite and growth rate of gilthead bream *Sparus aurata* L. J. Fish Biol. 20, 673–682.

Tillner, R., Rønnestad, I., Dhert, P., Ueberschär, B., 2014. The regulatory loop between gut cholecystokinin and tryptic enzyme activitcxuy in sea bass (*Dicentrarchus labrax*) larvae is influenced by different feeding regimes and trigger substances. Aquaculture 420-421, 139–146.

Tillner, R., Rønnestad, I., Harboe, T., Ueberschär, B., 2013. Hormonal control of tryptic enzyme activity in Atlantic cod larvae (*Gadus morhua*): Involvement of cholecystokinin during ontogeny and diurnal rhythm. Aquaculture 402-403, 133–140.

Ueberschär, B., 1995. The use of tryptic enzyme activity measurement as a nutritional condition index: Laboratory calibration data and field application. ICES mar. Sci. Symp.

Ueberschär, B., 1988. Determination of the nutritional condition of individual marine fish larvae by analyzing their proteolytic enzyme activites with a highly sensitive fluorescence technique. Meeresforsch. Rep. mar. Res. 32, 144–154.

Ueberschär, B., 1993. Measurement of proteolytic enzyme activity: significance and application in larval fish research, in: Physiological and Biochemical Aspects of Fish Development. 233–239.

Ueberschär, B., 2006. Kritische Zeiten für Fischlarven – Bestimmung der Trypsinaktivität zur Beurteilung des Ernährungszustands von Fischlarven in Labor- und Felduntersuchungen – ein Überblick, in: Verhandlungen Der Gesellschaft Für Ichtyologie, Band 5. 193–196.

Ueberschär, B., Pedersen, B.H., Hjelmeland, K., 1992. Quantification of trypsin with a radioimmunoassay in herring larvae (*Clupea harengus*) compared with a highly sensitive fluorescence technique to determine tryptic enzyme activity. Mar. Biol. 113, 469–473.

Valente, L.M.P., Moutou, K. a., Conceição, L.E.C., Engrola, S., Fernandes, J.M.O., Johnston, I. a., 2013. What determines growth potential and juvenile quality of farmed fish species? Rev. Aquac. 5, 168–193.

Van der Meeren, T., Mangor-Jensen, A., Pickova, J., 2007. The effect of green water and light intensity on survival, growth and lipid composition in Atlantic cod (*Gadus morhua*) during intensive larval rearing. Aquaculture 265, 206–217.

Webb, K., Holt, J., n.d. Cholecystokinin and trypsin responses of larval red drum (*Sciaenops Ocellatus*) to soluble components of rotifers (*Brachionus Plicatilis*) and Algae (*Isochrysis Galbana*) 79–89.

Yúfera, M., Darias, M.J., 2007. The onset of exogenous feeding in marine fish larvae. Aquaculture 268, 53–63.

Zambonino Infante, J.L., Cahu, C., 1994. Development and response to a diet change of some digestive enzymes in sea bass (*Dicentrarchus labrax*) larvae. Fish Physiol. Biochem. 12, 399–408.

Zambonino Infante, J.L., Cahu, C.L., 2001. Ontogeny of the gastrointestinal tract of marine fish larvae. Comp. Biochem. Physiol. Part C Toxicol. Pharmacol. 130, 477–487.

Zandee, D.I., Kluytmans, J.H., Zurburg, W., 1980. Seasonal variations in biochemical composition of *Mytilus edulis* with reference to energy metabolism and gametogenesis. Netherlands J. Sea Res. 14, 1–29.

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Appendix

Table 3: Mean values and standard deviations (SD) of tryptic enzyme activity of unfed and fed larvae (n = 6) at different sampling times during the diel rhythm experiment at 13 dph.

Date	Age [dph]	Treatment	Sampling time	Tryptic enzyme activity [hydrolyzed MCA nmol min^{-1} $larva^{-1}$]	SD
30.06.2014	13	fed with *Artemia*	06:30	0.79	0.26
30.06.2014	13	fed with *Artemia*	07:15	0.91	0.41
30.06.2014	13	fed with *Artemia*	08:00	1.41	0.87
30.06.2014	13	fed with *Artemia*	09:00	0.88	0.34
30.06.2014	13	fed with *Artemia*	10:00	1.21	0.36
30.06.2014	13	fed with *Artemia*	11:00	1.16	0.61
30.06.2014	13	fed with *Artemia*	12:00	0.74	0.27
30.06.2014	13	fed with *Artemia*	13:00	1.57	0.44
30.06.2014	13	fed with *Artemia*	14:00	1.59	0.65
30.06.2014	13	fed with *Artemia*	15:00	2.42	1.50
30.06.2014	13	fed with *Artemia*	16:00	0.61	0.26
30.06.2014	13	fed with *Artemia*	17:00	0.97	0.41
30.06.2014	13	fed with *Artemia*	18:00	1.21	0.44
30.06.2014	13	fed with *Artemia*	19:00	1.86	0.73
30.06.2014	13	fed with *Artemia*	20:00	0.93	0.43
30.06.2014	13	fed with *Artemia*	21:00	1.54	0.25
30.06.2014	13	fed with *Artemia*	22:00	2.82	1.00
30.06.2014	13	fed with *Artemia*	23:00	1.49	0.35
01.07.2014	14	fed with *Artemia*	06:30	1.43	0.35
30.06.2014	13	unfed	06:30	0.89	0.40
30.06.2014	13	unfed	07:15	1.29	0.93
30.06.2014	13	unfed	08:00	0.76	0.48
30.06.2014	13	unfed	09:00	0.83	0.27
30.06.2014	13	unfed	10:00	2.32	2.17
30.06.2014	13	unfed	11:00	1.59	1.61
30.06.2014	13	unfed	12:00	0.59	0.25
30.06.2014	13	unfed	13:00	1.23	0.29
30.06.2014	13	unfed	14:00	1.00	0.44
30.06.2014	13	unfed	15:00	1.34	0.38
30.06.2014	13	unfed	16:00	0.46	0.18
30.06.2014	13	unfed	17:00	0.56	0.09
30.06.2014	13	unfed	18:00	0.46	0.23
30.06.2014	13	unfed	19:00	1.05	0.71
30.06.2014	13	unfed	20:00	0.29	0.19
30.06.2014	13	unfed	21:00	0.55	0.21
30.06.2014	13	unfed	22:00	1.01	0.13
30.06.2014	13	unfed	23:00	0.36	0.16
01.07.2014	14	unfed	06:30	1.12	0.17

Table 4: Mean values and standard deviations (SD) of tryptic enzyme activity of unfed and fed larvae (n = 6) at different sampling times during the diel rhythm experiment at 20 dph.

Date	Age [dph]	Treatment	Sampling time	Tryptic enzyme activity [hydrolyzed MCA nmol min^{-1} $larva^{-1}$]	SD
07.07.2014	20	fed with *Artemia*	06:30	1.19	0.38
07.07.2014	20	fed with *Artemia*	07:15	2.23	0.77
07.07.2014	20	fed with *Artemia*	08:00	1.93	0.57
07.07.2014	20	fed with *Artemia*	09:00	2.78	0.96
07.07.2014	20	fed with *Artemia*	10:00	2.21	0.68
07.07.2014	20	fed with *Artemia*	11:00	3.18	1.31
07.07.2014	20	fed with *Artemia*	12:00	2.43	1.32
07.07.2014	20	fed with *Artemia*	13:00	3.10	1.19
07.07.2014	20	fed with *Artemia*	14:00	2.31	1.35
07.07.2014	20	fed with *Artemia*	15:00	3.87	1.01
07.07.2014	20	fed with *Artemia*	16:00	2.55	0.97
07.07.2014	20	fed with *Artemia*	17:00	4.93	1.76
07.07.2014	20	fed with *Artemia*	18:00	3.14	1.32
07.07.2014	20	fed with *Artemia*	19:00	4.87	2.50
07.07.2014	20	fed with *Artemia*	20:00	4.86	1.34
07.07.2014	20	fed with *Artemia*	21:00	7.13	1.90
07.07.2014	20	fed with *Artemia*	22:00	6.41	2.30
07.07.2014	20	fed with *Artemia*	23:00	7.22	1.92
08.07.2014	21	fed with *Artemia*	06:30	1.47	0.51
07.07.2014	20	unfed	06:30	1.74	0.38
07.07.2014	20	unfed	07:15	2.13	1.30
07.07.2014	20	unfed	08:00	1.56	0.64
07.07.2014	20	unfed	09:00	2.03	0.49
07.07.2014	20	unfed	10:00	1.43	0.45
07.07.2014	20	unfed	11:00	1.92	0.67
07.07.2014	20	unfed	12:00	1.59	0.63
07.07.2014	20	unfed	13:00	1.85	0.64
07.07.2014	20	unfed	14:00	1.35	0.74
07.07.2014	20	unfed	15:00	1.85	0.86
07.07.2014	20	unfed	16:00	1.01	0.43
07.07.2014	20	unfed	17:00	1.50	0.34
07.07.2014	20	unfed	18:00	1.02	0.35
07.07.2014	20	unfed	19:00	1.75	1.07
07.07.2014	20	unfed	20:00	1.04	0.30
07.07.2014	20	unfed	21:00	1.16	0.18
07.07.2014	20	unfed	22:00	1.15	0.59
07.07.2014	20	unfed	23:00	0.97	0.34
08.07.2014	21	unfed	06:30	1.77	0.43

Table 5: Mean values and standard deviations (SD) of tryptic enzyme activity of unfed and fed larvae (n = 6) at different sampling times during the diel rhythm experiment at 27 dph.

Date	Age [dph	Treatment	Sampling time	Tryptic enzyme activity [hydrolyzed MCA nmol min^{-1} $larva^{-1}$]	SD
14.07.2014	27	fed with *Artemia*	06:30	1.54	0.41
14.07.2014	27	fed with *Artemia*	07:15	2.62	0.69
14.07.2014	27	fed with *Artemia*	08:00	3.23	1.33
14.07.2014	27	fed with *Artemia*	09:00	5.53	1.45
14.07.2014	27	fed with *Artemia*	10:00	4.62	1.49
14.07.2014	27	fed with *Artemia*	11:00	6.32	4.08
14.07.2014	27	fed with *Artemia*	12:00	8.11	4.75
14.07.2014	27	fed with *Artemia*	13:00	7.43	7.17
14.07.2014	27	fed with *Artemia*	14:00	10.51	2.82
14.07.2014	27	fed with *Artemia*	15:00	8.09	5.85
14.07.2014	27	fed with *Artemia*	16:00	6.12	5.09
14.07.2014	27	fed with *Artemia*	17:00	9.67	6.38
14.07.2014	27	fed with *Artemia*	18:00	9.93	6.50
14.07.2014	27	fed with *Artemia*	20:00	6.17	3.46
14.07.2014	27	fed with *Artemia*	21:00	6.86	4.11
14.07.2014	27	fed with *Artemia*	22:00	6.52	4.17
14.07.2014	27	fed with *Artemia*	23:00	17.76	6.94
15.07.2014	28	fed with *Artemia*	06:30	2.45	0.80
14.07.2014	27	fed with *Artemia*	06:30	3.25	1.21
14.07.2014	27	unfed	07:15	2.97	1.36
14.07.2014	27	unfed	08:00	2.23	0.81
14.07.2014	27	unfed	09:00	2.99	0.59
14.07.2014	27	unfed	10:00	2.25	1.34
14.07.2014	27	unfed	11:00	2.94	0.97
14.07.2014	27	unfed	12:00	2.39	0.42
14.07.2014	27	unfed	13:00	2.34	1.28
14.07.2014	27	unfed	14:00	2.13	0.55
14.07.2014	27	unfed	15:00	2.33	0.61
14.07.2014	27	unfed	16:00	2.62	0.30
14.07.2014	27	unfed	17:00	2.21	0.61
14.07.2014	27	unfed	18:00	2.42	0.87
14.07.2014	27	unfed	19:00	1.33	0.44
14.07.2014	27	unfed	20:00	2.34	0.80
14.07.2014	27	unfed	21:00	2.96	0.66
14.07.2014	27	unfed	22:00	1.64	0.41
14.07.2014	27	unfed	23:00	1.54	0.44
15.07.2014	28	unfed	06:30	1.86	0.41

Table 6: Mean values and standard deviations (SD) of control larvae and larvae, which were exposed for 10, 20 and 30 min (n = 6) to *Artemia* rearing water (ARW) during the three experimental phases (Exp. phase) of the trigger experiment trials at 14 days post hatch (dph), 21 dph and 28 dph.

Treatment	Date	Age [dph]	Exp. phase	Time [min]	Tryptic enzyme activity [hydrolyzed MCA nmol min^{-1} $larva^{-1}$]	SD
ARW	01.07.2014	14	1	10	0.47	0.19
ARW	01.07.2014	14	1	20	1.07	0.32
ARW	01.07.2014	14	1	30	0.49	0.17
ARW	01.07.2014	14	2	10	0.77	0.23
ARW	01.07.2014	14	2	20	0.38	0.29
ARW	01.07.2014	14	2	30	0.27	0.16
ARW	01.07.2014	14	3	10	0.51	0.30
ARW	01.07.2014	14	3	20	0.43	0.14
ARW	01.07.2014	14	3	30	0.38	0.18
ARW	08.07.2014	21	1	10	1.60	0.84
ARW	08.07.2014	21	1	20	1.28	0.31
ARW	08.07.2014	21	1	30	1.51	0.91
ARW	08.07.2014	21	2	10	1.00	0.56
ARW	08.07.2014	21	2	20	0.95	0.38
ARW	08.07.2014	21	2	30	0.79	0.49
ARW	08.07.2014	21	3	10	1.32	0.95
ARW	08.07.2014	21	3	20	0.86	0.36
ARW	08.07.2014	21	3	30	1.22	0.27
ARW	15.07.2014	28	1	10	2.83	1.32
ARW	15.07.2014	28	1	20	3.85	2.75
ARW	15.07.2014	28	1	30	1.87	1.14
ARW	15.07.2014	28	2	10	2.42	1.05
ARW	15.07.2014	28	2	20	2.83	1.47
ARW	15.07.2014	28	2	30	2.03	0.68
ARW	15.07.2014	28	3	10	2.26	1.45
ARW	15.07.2014	28	3	20	1.24	0.39
ARW	15.07.2014	28	3	30	1.61	0.74
control	01.07.2014	14	1	10	0.49	0.16
control	01.07.2014	14	1	20	0.83	0.28
control	01.07.2014	14	1	30	0.58	0.27
control	01.07.2014	14	2	10	0.83	0.27
control	01.07.2014	14	2	20	0.54	0.27
control	01.07.2014	14	2	30	0.27	0.12
control	01.07.2014	14	3	10	0.76	0.31
control	01.07.2014	14	3	20	0.58	0.24
control	01.07.2014	14	3	30	0.49	0.30
control	08.07.2014	21	1	10	1.53	0.42
control	08.07.2014	21	1	20	1.32	0.91
control	08.07.2014	21	1	30	0.74	0.11
control	08.07.2014	21	2	10	0.97	0.27
control	08.07.2014	21	2	20	1.45	0.83
control	08.07.2014	21	2	30	1.06	0.25
control	08.07.2014	21	3	10	0.89	0.53
control	08.07.2014	21	3	20	0.87	0.35
control	08.07.2014	21	3	30	1.09	0.45
control	15.07.2014	28	1	10	1.56	0.63
control	15.07.2014	28	1	20	1.95	1.03
control	15.07.2014	28	1	30	2.47	1.13
control	15.07.2014	28	2	10	1.49	0.53
control	15.07.2014	28	2	20	2.13	0.67
control	15.07.2014	28	2	30	1.88	0.72
control	15.07.2014	28	3	10	1.60	0.49
control	15.07.2014	28	3	20	1.33	0.65
control	15.07.2014	28	3	30	1.73	0.95

Table 7: Mean values and standard deviations (SD) of control larvae and larvae, which were exposed for 10, 20 and 30 min (n = 6) to a mixture of betaine, alanine, ariginine and glycine (BAAG) during the three experimental phases (Exp. phase) of the trigger experiment trials at 14 days post hatch (dph), 21 dph and 28 dph.

Treatment	Date	Age [dph]	Exp. phase	Time [min]	Tryptic activity [hydrolyzed MCA nmol min^{-1} $larva^{-1}$]	SD
BAAG	01.07.2014	14	1	10	1.20	0.24
BAAG	01.07.2014	14	1	20	0.81	0.42
BAAG	01.07.2014	14	1	30	0.83	0.28
BAAG	01.07.2014	14	2	10	0.54	0.12
BAAG	01.07.2014	14	2	20	0.84	0.57
BAAG	01.07.2014	14	2	30	0.54	0.21
BAAG	01.07.2014	14	3	10	0.57	0.24
BAAG	01.07.2014	14	3	20	1.02	0.38
BAAG	01.07.2014	14	3	30	0.48	0.19
BAAG	08.07.2014	21	1	10	2.32	0.97
BAAG	08.07.2014	21	1	20	1.73	0.63
BAAG	08.07.2014	21	1	30	1.76	0.78
BAAG	08.07.2014	21	2	10	1.84	0.62
BAAG	08.07.2014	21	2	20	2.42	1.59
BAAG	08.07.2014	21	2	30	1.28	0.66
BAAG	08.07.2014	21	3	10	1.86	0.28
BAAG	08.07.2014	21	3	20	1.07	0.48
BAAG	08.07.2014	21	3	30	0.98	0.39
BAAG	15.07.2014	28	1	10	3.03	1.13
BAAG	15.07.2014	28	1	20	2.94	2.29
BAAG	15.07.2014	28	1	30	3.62	2.19
BAAG	15.07.2014	28	2	10	3.21	1.11
BAAG	15.07.2014	28	2	20	4.23	2.85
BAAG	15.07.2014	28	2	30	4.07	2.05
BAAG	15.07.2014	28	3	10	3.54	2.67
BAAG	15.07.2014	28	3	20	2.47	1.47
BAAG	15.07.2014	28	3	30	2.02	1.08
control	01.07.2014	14	1	10	0.68	0.12
control	01.07.2014	14	1	20	0.69	0.20
control	01.07.2014	14	1	30	0.80	0.30
control	01.07.2014	14	2	10	0.30	0.11
control	01.07.2014	14	2	20	0.97	0.53
control	01.07.2014	14	2	30	0.68	0.29
control	01.07.2014	14	3	10	0.47	0.17
control	01.07.2014	14	3	20	0.68	0.20
control	01.07.2014	14	3	30	0.71	0.52
control	08.07.2014	21	1	10	2.26	0.97
control	08.07.2014	21	1	20	1.84	0.68
control	08.07.2014	21	1	30	1.17	0.55
control	08.07.2014	21	2	10	1.47	0.87
control	08.07.2014	21	2	20	1.52	0.95
control	08.07.2014	21	2	30	1.00	0.55
control	08.07.2014	21	3	10	1.49	0.99
control	08.07.2014	21	3	20	1.54	1.14
control	08.07.2014	21	3	30	1.54	0.48
control	15.07.2014	28	1	10	2.27	0.71
control	15.07.2014	28	1	20	2.90	2.47
control	15.07.2014	28	1	30	4.55	1.51
control	15.07.2014	28	2	10	2.70	1.04
control	15.07.2014	28	2	20	2.00	0.82
control	15.07.2014	28	2	30	2.60	1.24
control	15.07.2014	28	3	10	2.57	0.77
control	15.07.2014	28	3	20	2.43	1.04
control	15.07.2014	28	3	30	1.66	0.61

Table 8: Mean values and standard deviations (SD) of control larvae and larvae, which were exposed for 10, 20 and 30 min (n = 6) to a mixture of glycine and betaine (GB) during the three experimental phases (Exp. phase) of the trigger experiment trials at 15 days post hatch (dph), 22 dph, 29 dph and 36 dph.

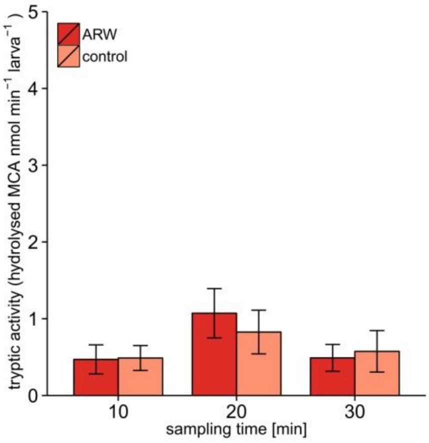
Treatment	Date	Age [dph]	Exp. phase	Time [min]	Tryptic activity [hydrolyzed MCA nmol min^{-1} $larva^{-1}$]	SD
GB	02.07.2014	15	1	10	0.63	0.34
GB	02.07.2014	15	1	20	0.37	0.10
GB	02.07.2014	15	1	30	0.85	0.19
GB	02.07.2014	15	2	10	0.72	0.37
GB	02.07.2014	15	2	20	0.57	0.16
GB	02.07.2014	15	2	30	0.40	0.17
GB	02.07.2014	15	3	10	0.52	0.29
GB	02.07.2014	15	3	20	0.45	0.16
GB	02.07.2014	15	3	30	0.36	0.12
GB	09.07.2014	22	1	10	0.88	0.19
GB	09.07.2014	22	1	20	0.85	0.32
GB	09.07.2014	22	1	30	1.09	0.33
GB	09.07.2014	22	2	10	0.73	0.26
GB	09.07.2014	22	2	20	1.11	0.44
GB	09.07.2014	22	2	30	0.67	0.47
GB	09.07.2014	22	3	10	0.98	0.43
GB	09.07.2014	22	3	20	1.11	0.88
GB	09.07.2014	22	3	30	1.69	0.65
GB	16.07.2014	29	1	10	2.51	0.59
GB	16.07.2014	29	1	20	1.50	0.72
GB	16.07.2014	29	1	30	2.30	1.61
GB	16.07.2014	29	2	10	1.74	0.53
GB	16.07.2014	29	2	20	2.06	0.88
GB	16.07.2014	29	2	30	3.21	2.01
GB	16.07.2014	29	3	10	2.07	0.92
GB	16.07.2014	29	3	20	2.50	1.83
GB	16.07.2014	29	3	30	1.62	0.30
GB	23.07.2014	36	1	10	4.42	1.88
GB	23.07.2014	36	1	20	2.90	1.31
GB	23.07.2014	36	1	30	2.38	0.99
GB	23.07.2014	36	2	10	3.52	2.36
GB	23.07.2014	36	2	20	2.00	1.13
GB	23.07.2014	36	2	30	2.05	0.69
GB	23.07.2014	36	3	10	2.27	0.44
GB	23.07.2014	36	3	20	2.98	1.20
GB	23.07.2014	36	3	30	2.45	0.49
control	02.07.2014	15	1	10	0.47	0.23
control	02.07.2014	15	1	20	0.47	0.17
control	02.07.2014	15	1	30	0.67	0.36
control	02.07.2014	15	2	10	0.60	0.14
control	02.07.2014	15	2	20	0.53	0.20
control	02.07.2014	15	2	30	0.66	0.13
control	02.07.2014	15	3	10	0.45	0.18
control	02.07.2014	15	3	20	0.43	0.28
control	02.07.2014	15	3	30	0.55	0.24
control	09.07.2014	22	1	10	1.10	0.62
control	09.07.2014	22	1	20	0.93	0.55
control	09.07.2014	22	1	30	1.12	0.50
control	09.07.2014	22	2	10	1.03	0.59
control	09.07.2014	22	2	20	0.85	0.41
control	09.07.2014	22	2	30	0.83	0.44
control	09.07.2014	22	3	10	1.02	0.52
control	09.07.2014	22	3	20	0.88	0.44
control	09.07.2014	22	3	30	1.15	0.72

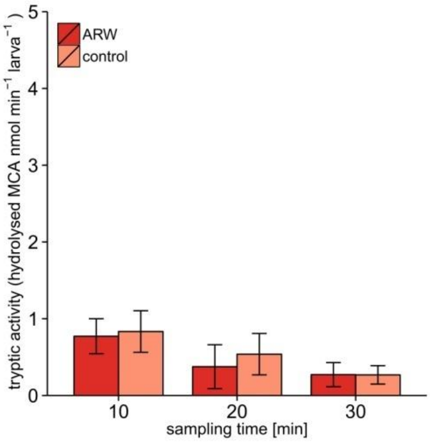
Table 8 continued.

Treatment	Date	Age [dph]	Exp. phase	Time [min]	Tryptic activity [hydrolyzed MCA nmol min^{-1} $larva^{-1}$]	SD
control	16.07.2014	29	1	10	1.59	0.40
control	16.07.2014	29	1	20	2.20	0.81
control	16.07.2014	29	1	30	1.96	0.33
control	16.07.2014	29	2	10	2.38	0.81
control	16.07.2014	29	2	20	1.81	0.98
control	16.07.2014	29	2	30	3.04	2.11
control	16.07.2014	29	3	10	1.97	0.59
control	16.07.2014	29	3	20	1.41	0.32
control	16.07.2014	29	3	30	2.14	0.90
control	23.07.2014	36	1	10	2.47	0.96
control	23.07.2014	36	1	20	2.29	0.88
control	23.07.2014	36	1	30	2.48	1.10
control	23.07.2014	36	2	10	3.83	2.22
control	23.07.2014	36	2	20	1.92	0.59
control	23.07.2014	36	2	30	2.49	0.90
control	23.07.2014	36	3	10	3.02	1.29
control	23.07.2014	36	3	20	3.56	2.16
control	23.07.2014	36	3	30	2.30	0.64

Table 9: Mean values and standard deviations (SD) of control larvae and larvae, which were exposed for 10, 20 and 30 min (n = 6) to an extract of *Mytilus edulis* (MY) during the three experimental phases (Exp. phase) of the trigger experiment trials at 15 days post hatch (dph), 22 dph and 29 dph.

Treatment	Date	Age [dph]	Exp. phase	Time [min]	Tryptic activity [hydrolyzed MCA nmol min^{-1} $larva^{-1}$]	SD
MY	02.07.2014	15	1	10	0.51	0.20
MY	02.07.2014	15	1	20	0.97	0.15
MY	02.07.2014	15	1	30	0.49	0.15
MY	02.07.2014	15	2	10	0.45	0.12
MY	02.07.2014	15	2	20	0.80	0.19
MY	02.07.2014	15	2	30	0.69	0.14
MY	02.07.2014	15	3	10	1.12	0.32
MY	02.07.2014	15	3	20	0.77	0.32
MY	02.07.2014	15	3	30	0.60	0.19
MY	09.07.2014	22	1	10	1.16	0.64
MY	09.07.2014	22	1	20	1.33	0.80
MY	09.07.2014	22	1	30	1.05	0.45
MY	09.07.2014	22	2	10	1.17	0.60
MY	09.07.2014	22	2	20	0.83	0.60
MY	09.07.2014	22	2	30	0.84	0.37
MY	09.07.2014	22	3	10	2.89	0.61
MY	09.07.2014	22	3	20	1.05	0.40
MY	09.07.2014	22	3	30	1.42	0.39
MY	16.07.2014	29	1	10	2.25	1.62
MY	16.07.2014	29	1	20	1.94	0.48
MY	16.07.2014	29	1	30	1.89	0.55
MY	16.07.2014	29	2	10	1.19	0.51
MY	16.07.2014	29	2	20	1.89	0.95
MY	16.07.2014	29	2	30	1.79	0.54
MY	16.07.2014	29	3	10	1.99	0.49
MY	16.07.2014	29	3	20	1.86	0.96
MY	16.07.2014	29	3	30	1.12	0.17
control	02.07.2014	15	1	10	0.84	0.31
control	02.07.2014	15	1	20	0.75	0.27
control	02.07.2014	15	1	30	0.53	0.31
control	02.07.2014	15	2	10	0.53	0.35
control	02.07.2014	15	2	20	0.90	0.29
control	02.07.2014	15	2	30	0.71	0.23
control	02.07.2014	15	3	10	0.91	0.48
control	02.07.2014	15	3	20	0.48	0.16
control	02.07.2014	15	3	30	0.53	0.08
control	09.07.2014	22	1	10	1.20	0.43
control	09.07.2014	22	1	20	1.41	0.51
control	09.07.2014	22	1	30	0.93	0.37
control	09.07.2014	22	2	10	0.98	0.61
control	09.07.2014	22	2	20	1.35	0.58
control	09.07.2014	22	2	30	1.03	0.70
control	09.07.2014	22	3	10	1.76	0.56
control	09.07.2014	22	3	20	1.45	0.42
control	09.07.2014	22	3	30	1.40	0.36
control	16.07.2014	29	1	10	2.11	1.10
control	16.07.2014	29	1	20	2.64	1.09
control	16.07.2014	29	1	30	1.63	0.84
control	16.07.2014	29	2	10	1.16	0.50
control	16.07.2014	29	2	20	1.67	0.69
control	16.07.2014	29	2	30	2.41	0.90
control	16.07.2014	29	3	10	1.79	0.61
control	16.07.2014	29	3	20	1.31	0.85
control	16.07.2014	29	3	30	1.14	0.29





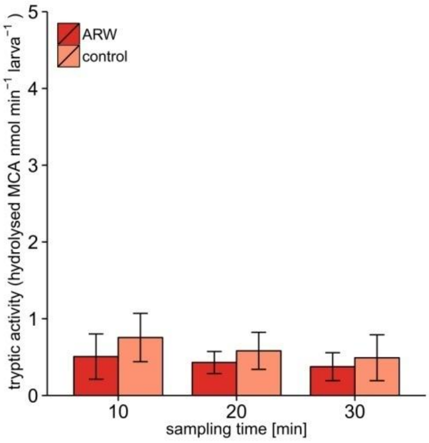
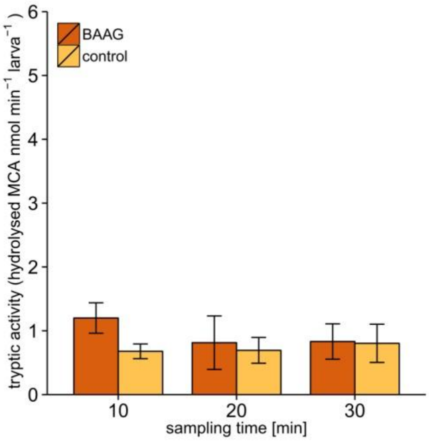
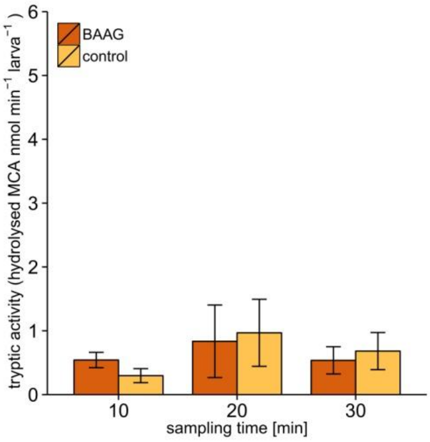


Figure 20: Tryptic enzyme activity of sea bass larvae during the three experimental phases (a - c) of trigger experiment with ARW (*Artemia* rearing water) as trigger solution at 14 dph. Sampling of larvae, which were exposed to ARW, and control larvae (without any trigger) was carried out after 10, 20 and 30 min. Values (a - c) are means and standard deviations of 6 larvae.





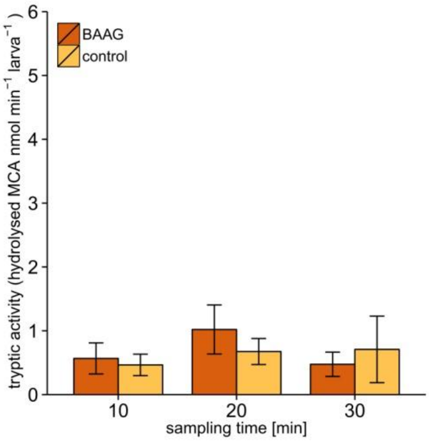
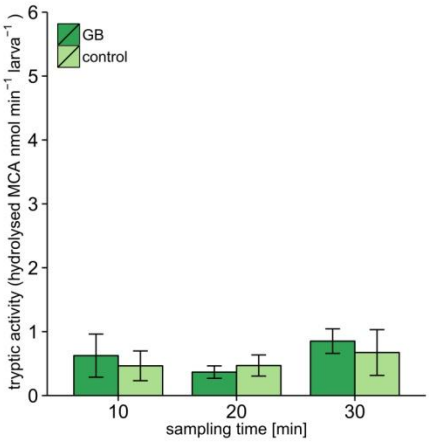
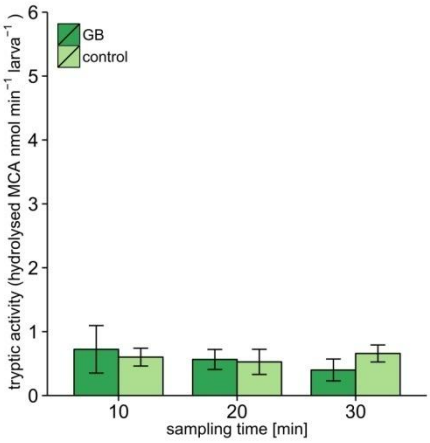


Figure 21: Tryptic enzyme activity of sea bass larvae during the three experimental phases (a - c) of trigger experiment trial with BAAG (betaine, alanie, arginine and glycine) as trigger solution at 14 dph. Sampling of larvae, which were exposed to BAAG, and control larvae (without any trigger) was carried out after 10, 20 and 30 min. Values (a - c) are means and standard deviations of 6 larvae.





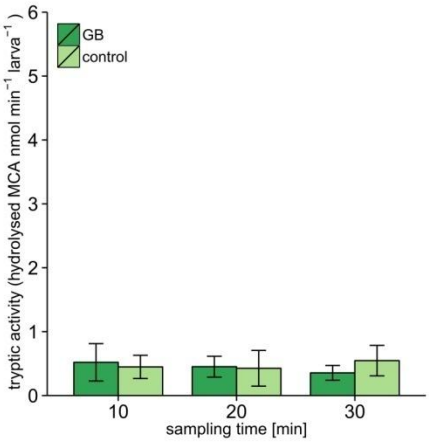
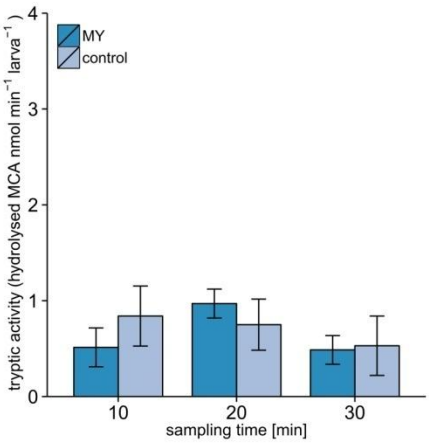
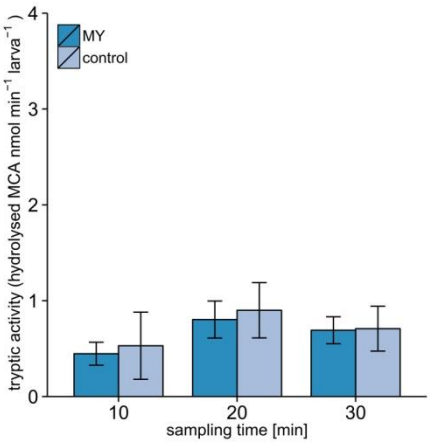


Figure 22: Tryptic enzyme activity of sea bass larvae during the three experimental phases (a - c) of trigger experiment trial with GB (glycine and betaine) as trigger solution at 15 dph. Sampling of larvae, which were exposed to GB, and control larvae (without any trigger) was carried out after 10, 20 and 30 min. Values (a - c) are means and standard deviations of 6 larvae.





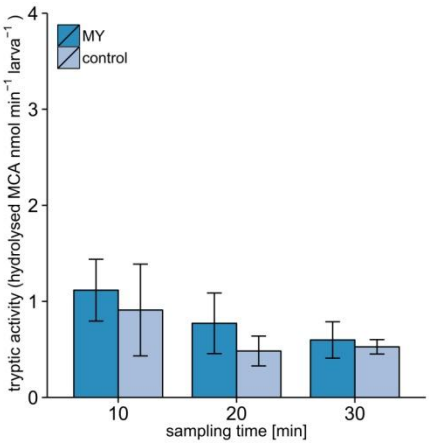
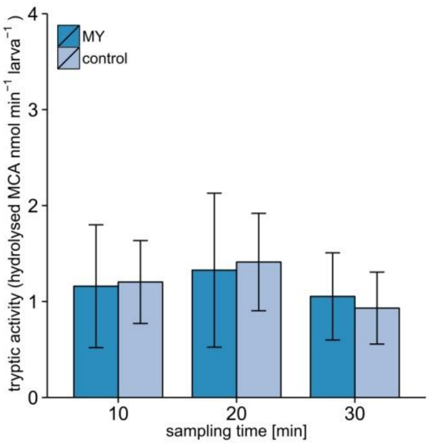
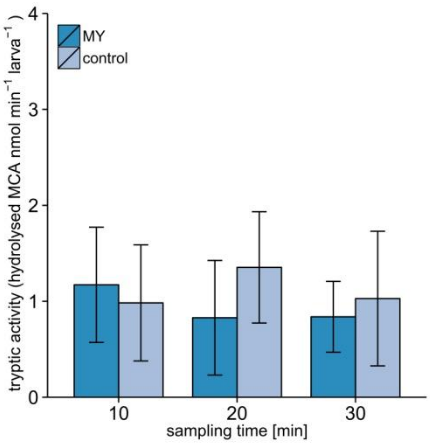


Figure 23: Tryptic enzyme activity of sea bass larvae during the three experimental phases (a - c) of trigger experiment trial with MY (extract of *Mytilus edulis*) as trigger solution at 15 dph. Sampling of larvae, which were exposed to MY, and control larvae (without any trigger) was carried out after 10, 20 and 30 min. Values (a - c) are means and standard deviations of 6 larvae.





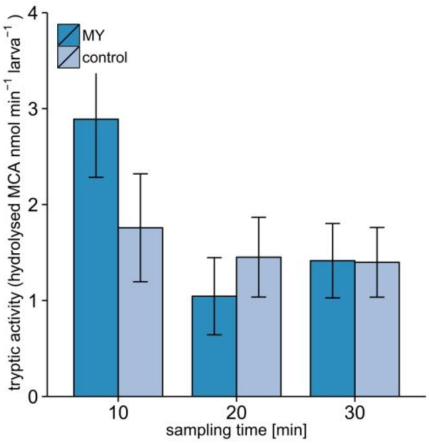
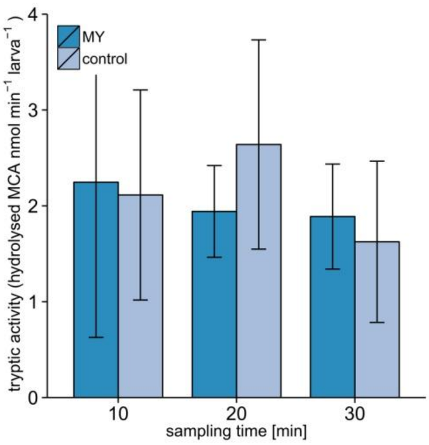
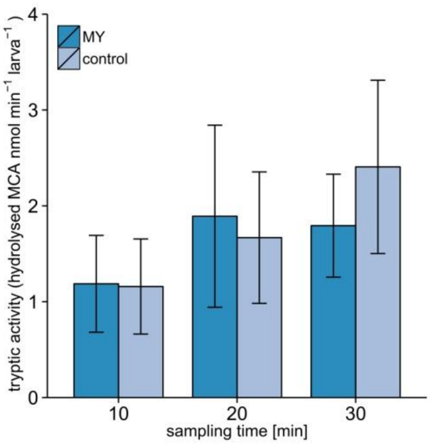


Figure 24: Tryptic enzyme activity of sea bass larvae during the three experimental phases (a - c) of trigger experiment trial with MY (extract of *Mytilus edulis*) as trigger solution at 21 dph. Sampling of larvae, which were exposed to MY, and control larvae (without any trigger) was carried out after 10, 20 and 30 min. Values (a - c) are means and standard deviations of 6 larvae.





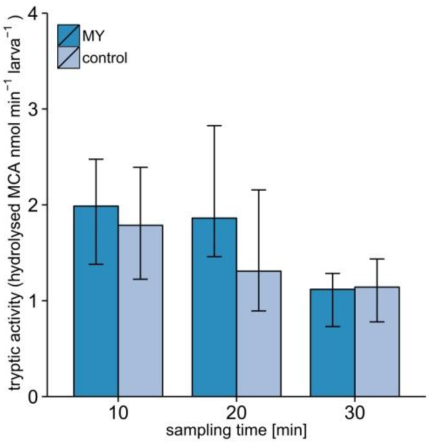


Figure 25: Tryptic enzyme activity of sea bass larvae during the three experimental phases (a - c) of trigger experiment trial with MY (extract of *Mytilus edulis*) as trigger solution at 28 dph. Sampling of larvae, which were exposed to MY, and control larvae (without any trigger) was carried out after 10, 20 and 30 min. Values (a - c) are means and standard deviations of 6 larvae.

Hiermit bestätige ich, dass die vorliegende Arbeit von mir selbständig verfasst wurde und ich keine anderen als die angegebenen Hilfsmittel – insbesondere keine im Quellenverzeichnis nicht benannten Internet–Quellen– benutzt habe und die Arbeit von mir vorher nicht einem anderen Prüfungsverfahren eingereicht wurde. Die eingereichte schriftliche Fassung entspricht der auf dem elektronischen Speichermedium. Ich bin damit einverstanden, dass die Masterarbeit veröffentlicht wird.

Hamburg, den 31. März 2015 _____