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Towards optimisation of fish larval nutrition: the effects of new components in microdiet composition and feeding practices on larval digestive physiology

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For my family

‘‘What we know is a drop;

What we don’t know is an ocean’’

Isaac Newton

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General Introduction

Global fisheries production (excluding aquatic plants) reached in total 167.2 million tons in 2014, with 93.4 million tons from capture and 73.8 million tons from aquaculture (FAO, 2016). In terms of food supply, the world's population consumed more farmed fish than wild-caught fish for the first time in 2014. To maintain baseline consumption in every country, increased aquaculture production is seen as alternative to meet the demand of food for the predicted 9 billion people in 2050 (FAO, 2016).

As world consumption of seafood continues to increase, the farming of marine species has become a rapidly growing industry (Joffre et al., 2017). Sustainable growth of marine aquaculture industry requires fry in a reliable quality and quantity and there are several challenges hindering the rapid advancement of marine fish larvae culture. For example, marine fish larvae weigh less at hatch, show higher mortality rates, perform slower growth rates (comparison between sea bass, sea bream and rainbow trout, carp (FAO, 2018, species profiles), have higher metabolic requirements such as energy needs, oxygen uptake (Chabot et al., 2016), and in some cases have longer duration of larval-stage than some freshwater aquaculture fish larvae (Houde, 1994). Additionally, the digestive track of marine fish larvae is poorly developed at hatching and beyond (Zambonino Infante et al., 2008), thus affecting survival and growth rates. Despite many research efforts on the advancement of marine fish, high mortalities during larval production is still considered as a bottleneck for aquaculture production. High mortalities are apparently related to the poor performance of the digestive tract in early larval stages. Thus, the quality of starter feeds which have to meet exactly the physiological and digestive capacities of the early larval stages is of major significance in the optimization process to improve survival rates and growth performance. Knowledge on fish larval nutritional requirements, feeding managements, their digestive capacities and their implications for the formulation of microdiets are not yet fully understood (Conceição and Tandler, 2018; Hamre et al., 2013; Rønnestad and Morais, 2008; Yufera, 2018). The reason for the considerable research effort on these topics is obvious: feed constitutes a large portion of the costs in hatcheries and cost-effective production of high quality marine fish larvae are depending on deep knowledge of larval nutritional and digestive physiology. Therefore, optimising diet formulation and feed management strategies towards specific requirements of the larval stages of marine fish are key factors for improving the production efficiency and sustainability of marine aquaculture.

The digestive system of marine fish larvae

Most common marine fish larvae species in aquaculture, sea bass is a typical example, lack a functional stomach at onset of first feeding and undergo major developmental changes in the digestive tract until metamorphosis (Cahu and Zambonino Infante, 1994). Larvae of sea bass, for example, can produce some digestive enzymes early in their life cycle: trypsin at hatching; chymotrypsin, esterase and amylase from day 5; and lipase by day 15 (Rønnestad et al., 2013; Walford and Lam, 1993). A peptic activity (gastric glands secretion) can be measured in sea bass larvae after day 25- 30 post hatching (People Le Ruyet et al., 1993; Yúfera and Darias, 2007). Measurements of peptic digestive enzyme activity, for example, reflect responses to nutritional condition occurring at the cellular level. The secretion of trypsin is known to occur in response to feed ingestion, quality and amount hydrolysis of nutrients (Applebaum and Holt, 2003). Most of the proteolytic digestive enzymes are produced in the pancreas and are released into the digestive tract. Among the pancreatic enzymes, trypsin (and its precursor trypsinogen) is the most significant protease in the early larval stages and is considered to be a key enzyme in the digestive process which also reflects nutritional status of fish larvae (Rønnestad et al., 1999; Ueberschär, 1995; Zambonino Infante and Cahu, 2001). For instance, trypsin levels rise throughout the yolk-sac stage, but after the yolk has been absorbed, they may fall rapidly before rising again in older larvae of Atlantic halibut (Hamre et al., 2007). This critical period was suggested to be considered as a ’four-phase model’ in the ontogenetic development of tryptic activity in marine fish larvae from hatching to metamorphosis (Rønnestad et al., 2013; Ueberschär, 1993). Tryptic enzyme activity measurement was introduced by Ueberschär (1988), who applied a highly sensitive fluorescence technique for measurement of tryptic activity in individual larval fish. In this study, the author found out that the increase in fluorescence due to the release of the fluorescence molecule is proportional to the amount of trypsin in the sample. Accordingly, it is already established that by using a fluorogenic substrate, tryptic enzyme activity in individual larvae could be measured and related to nutritional condition (Ueberschär and Clemmesen, 1992). In this thesis, the observation of tryptic enzyme activity during feeding periods was used as an indicator to optimize and adapt the feeding regimes and feeds to the digestive capacity of the sea bass and sea bream larvae.

Issues, constraints, challenges and requirements for a sustainable development in marine fish larval culture

It is still a common practice to use rotifers and *Artemia* as live feed in the intensive rearing of marine fish larvae, at least during the first few weeks after hatching. The application of live feeds have several advantages, for examples, as a behavioral stimulus to attack particles and are suitable for transferring necessary nutrients after enrichment for feeding larvae due to small size of larval mouth (Zambonino Infante and Cahu, 2001). However, there are many issues and problems associated with sourcing and use of live feed organisms and to maintain standardized continuous production in hatcheries. Some of the issues and challenges in the current use of live prey for larviculture include an expensive, time-consuming process. In addition, the use of live feeds is not always reliable (unpredictable quality and quantity) (Faulk and Holt, 2009), mainly associated with the availability and price fluctuations (Callan et al., 2003), poor hygienic conditions and high bacterial loads (Olafsen, 2001; Olsen et al., 2000), low contents of omega-3 fatty acids and low levels of specific vitamins and minerals (Bonaldo et al., 2011). In order to meet the requirements for fatty acids and nutrients, rotifer and *Artemia* are commonly enriched with highly unsaturated fatty acids (HUFA), eicosapentaenoic acid (EPA: 20:5n-3), docosahexanoic acid (DHA: 22:6n-3), vitamins (C and E), minerals (iodine and selenium), probiotics, lipid emulsions, amino acids (methionine and lysine) (Dhert et al., 2001; Harel et al., 2002; Monroig et al., 2006) in the culture of marine fish larvae (Izquierdo, 1996). However, even with an enrichment, the nutritional values of rotifers and *Artemia* may still be inferior as compared to copepod nauplii (Zeng et al., 2018). The limited nutrional value of using rotifer and *Artemia* may partly explain why fish larvae in intensive farming grow more slowly and suffer higher mortalities and deformities than larvae kept under culture conditions fed with copepods (Hamre et al., 2007). Copepod nauplii, not only have nutritional profiles that meet larval fish requirements (Evjemo et al., 2003; Rønnestad et al., 1999), but also contain high levels of digestive enzymes (Conceição et al., 2010b) and show the capability of producing appetite stimulatory effects on larvae (Olivotto et al., 2011). However, the use of copepods in commercial hatcheries is still limited, largely due to costs and difficulties in their intensive culture (Stottrup, 2006).

In the last two decades, the feed industry worked intensively to develop a formulated diet (microdiet), usually particle sizes ranging between 50μm and 700μm, for first-feeding of marine

fish larvae in order to substitute live feed completely or as early as possible (Kolkovski et al., 2009). A complete microdiet which can replace live prey or allow to have early weaning and optimization of microdiets in feeding regimes can have benefits on reducing hatchery cost. For the development of more cost-efficient microdiets, more detailed knowledge on the nutritional requirements and limitations for culturing of farmed fish larvae is needed. Microdiets have several positive features such as availability, storability and having stable nutritive quality. However, other features apart from the nutritional value are important: proper size, attractive colour and smell, high water-stability (minimal leaching of nutrients), high digestibility and proper suspension in the water column (Burnell, 2013; Rathore et al., 2016). The main obstacles for marine fish larvae feed with a microdiet are nutrient digestion, assimilation and ingestion (Kolkovski et al., 1997a; Koven et al., 2001; Yúfera et al., 2005) (Fig.GI-1). Since fish larvae have an immature digestive system at early developmental stages, development of microdiet should suit the capacity of fish larvae for digestion and absorption of nutrients (Kjørsvik et al., 2004; Yúfera et al., 2000).

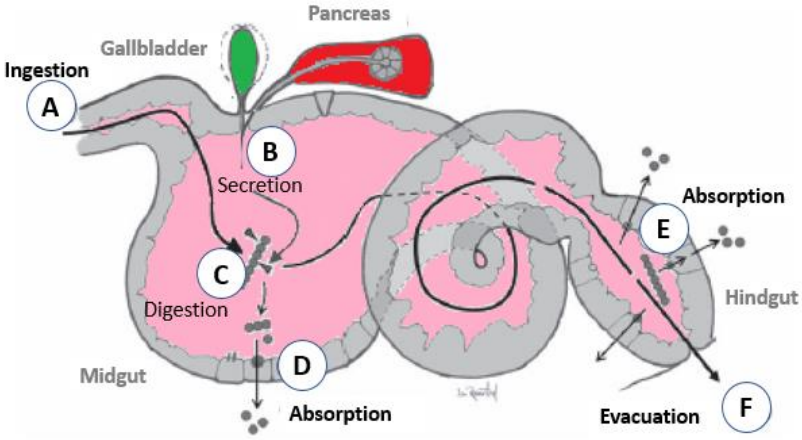

Fig.GI-1. Digestion and absorption of dietary proteins are involved in coordination of a range of basic processes in the digestive tract. In agastric fish larvae, these includes enzymatic secretions (B), digestion (C), absorption (D, E), and motility (including evacuation, (F). (Conceição et al., 2011).

Feeding practices are as important as nutritionally adequate and balanced microdiets in fish larvae culture. Feeding practices are dependent on decisions determined by farm managers detailing how much, how often, when, how, and where to feed (Kaushik, 1996; Lee et al., 2015). Feeding levels such as underfeeding in terms of supply of essential nutrients in adequate

quantities to meet needs for growth and maintenance influence the nutritional status of fish larvae. This could also affect directly or indirectly their growth performance (Harpaz et al., 2005; Verreth et al., 1993). Numerous publications addressing recommended feeding practices for various fish larvae species are available (Barnabé and Guissi, 1994; Brown et al., 2003; Curnow et al., 2006; Dou et al., 2000; Engrola et al., 2009; Fletcher et al., 2007; Holt, 1993; Kestemont et al., 2007; Kuhlmann et al., 1981; Papandroulakis et al., 2000). However, sparse research has examined the effects of feeding regimes in relation to fish larval digestive physiology. Feeding rates (food intake per hour) are one of the most important success factors for fish larval production (Conceição and Tandler, 2018). There is evidence that prolonged feeding periods and high rations decrease digestive efficiency (Rabe and Brown, 2000). Chapter I attempts to provide an overview of the effects of feeding practices on the digestive enzyme capacity of fish larvae. The effects of feeding time on tryptic enzyme activity as well as different feeding treatments (live feeds and microdiet) are also discussed.

In a similar manner, a look at the nutrient composition of microdiets in relation to early larval nutrition is extremely important for long term survival and growth of fish. Deficiencies in nutritional requirement of marine fish larvae could not only affect larval survival and growth, but also general health and normal skeleton formations (de Vareilles et al., 2012; Fernández et al., 2008). Fish larvae require essential amino acids (Rønnestad et al., 1999), fatty acids, phospholipids, energy, vitamins and minerals (Hamre et al., 2013; Moren et al., 2011). Glencross et al. (2007) summarised this as, "a feed is only as good as its ingredients". The major feed ingredient source in microdiets is fish meal. Fish meal is a highly nutritious feed ingredient, primarily used as high quality protein source as it has an excellent amino acid profile, is very palatable and digestible, is a rich source of energy, contain essential fatty acids, vitamins and minerals and has low levels of antinutritional factors (Gatlin et al., 2007; Hardy, 2010). However, digestibility is a key factor in evaluating the potential of an ingredient for use in fish larvae nutrition (Cho and Kaushik, 1990; Allan et al., 2000). Microdiets containing fishmeal could be difficult for marine fish larvae to digest compared to live feeds. Thus, Calanus meal from *Calanus finmarchicus* which is highly similar to the natural diet of marine fish larvae could be an alternative natural ingredient in microdiets. In Chapter III of this thesis, we investigated the use of Calanus meal as alternative ingredient to fishmeal in feeding larvae of sea bass. In particular, Calanus meal contain long chain omega-3 fatty acids, mostly incorporated in

phospholipids, as well as astaxanthin. Its nutrient composition, which fits to the requirements of early larval nutrition, could mimic the palatability of live feed and also provide not only macronutrients and micronutrients but also in terms of digestibility.

Thesis outline and research questions

The criteria for succeeding in high quality marine fish larvae culture are complex and usually a combination of external (environmental and nutritional) and internal (molecular, developmental, physiological, behavioral, genetic) factors (Conceição and Tandler, 2018). A deeper knowledge in fish nutrition, physiology, balanced nutritional composition with appropriate feeding management could give the better growth, survival and optimal development of larvae over the critical period where most mortalities occurs. This PhD thesis aims to deal with three important segments in optimization of marine fish propagation answer three main components: *feeding practices*, such as feeding time and frequency during early larval stages and adaptation of feeding regimes to larval digestive capacity (Chapter I), *substances* which have the potential to trigger and eventually enhance the proteolytic digestive capacity (Chapter II) and *optimisation of microdiet formulations* (Chapter III) for reaching a level where live feed can be substituted or completely be replaced.

Chapter I

The aim of the first chapter of this work was to evaluate the impact of different dietary treatments on the diurnal digestive tryptic enzyme activity in gilthead sea bream (*Sparus aurata*) larvae. In order to evaluate optimum feeding time and frequency, several short-term experiments were conducted at different ages with different dietary treatments such as traditionally used live feeds (rotifers and *Artemia*) and microdiet.

Following specific topics were investigated in Chapter I:

- *Can feeding amount, time, number of meals be elasticized according to diurnal rhythms of digestive capacity of fish larvae?*
- *What are the optimal feeding times and frequency of sea bream larvae in culture conditions under special consideration of tryptic enzyme activity at different larval stages?*
- *Is there any difference in diurnal digestive enzyme activity when larvae fed with live feed or microdiet?*

Chapter II

The aim of the second chapter of this work was to investigate the effect of different substances that potentially can enhance the trypsinogen production and secretion in various age stages of sea bass larvae. The substances under evaluation here were selected as potential ingredients to be used in the formulation of microdiets for marine fish larvae.

Following specific issues were addressed in Chapter II:

- *What trigger substances could be used in European seabass microdiets to enchance digestibility?*
- *Can small volumes of dissolved trigger substances which are administered directly into the gut of fish larvae by tube-feeding technique induce tryptic enzyme activity?*
- *What are the benefits and limitations of tube-feeding technique?*

Chapter III

The third chapter of this work focused on the potential of a product from freeze-dried Calanus *(Calanus finmarchicus)* meal as a major component in microdiet for sea bass larvae. The main goal of this experiment was to compare and evaluate the ontogenetic development of tryptic enzyme activity in larval sea bass either fed live feed, a commercial microdiet or the microdiet containing the Calanus meal as major ingredient.

Following specific research questions aimed to be answered in Chapter III:

- *Is Calanus meal an alternative protein source in microdiet for marine fish larvae?*
- *How does the replacement of live feed by formulated microdiet affect growth performance, survival and tryptic enzyme activity in sea bass larvae?*
- *What are the effects of different enzymes addition (trypsin and pancreatin) in microdiets for sea bass larvae on their digestive enzyme capacity, growth and survival rate?*
- *Can Calanus meal decrease/replace fishmeal dependence in European seabass larval microdiet?*

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CHAPTER I

Diurnal patterns of tryptic enzyme activity under different feeding regimes in gilthead sea bream (*Sparus aurata*) larvae

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Abstract

Knowledge about the dynamic of the diurnal digestive enzyme capacity during early larval stages is crucial for the determination of appropriate feeding schedules, such as feeding time and frequency. However, only few data are available for some species. In this study, several short-term (20 h) diurnal rhythm experiments were conducted under 24 h light conditions to assess the impact of different dietary treatments on the diurnal patterns of tryptic enzyme activity in gilthead sea bream (*Sparus aurata*) larvae. Four different feeding regimes (groups fed with rotifers *Brachionus plicatilis* (R), groups fed with Artemia (A), groups fed with rotifers and Artemia (RA), groups fed with MicroDiet (MD)) were assessed at four different age stages (21, 26, 34 and 44 dph). Experimental groups were fed three times a day at 07:15, 14:15 and 22:15, and only the group fed with MD was fed every 15 minutes with an automatic feeding system. Additionally, in each experiment subgroups of larvae deprived of food were evaluated as control. Diurnal variation of tryptic activity in fed sea bream larvae groups showed a clear response on the administration of feed with increasing tryptic enzyme activity response after the feeding events. However, the activities in the morning and noon revealed relatively high levels in comparison to the activity after feeding in the evening. In contrast, tryptic enzyme activity remained significantly lower in larvae deprived of food compared to the fed groups throughout the day. Larvae in group A and MD at 44 dph showed a similar diurnal pattern in tryptic enzyme activity although group MD has been fed continuously. The results suggest that no matter what kind of diet was applied sea bream larvae have a limited digestive capacity at some point in time during the day.

Key words: Sea bream larvae; *Sparus aurata*; Trypsin; Diurnal rhythm; Digestive capacity

1. Introduction

It is a common phenomenon that under natural conditions a circadian rhythm in feed intake is existing in the larval and early juvenile stages of fish (Boujard and Leatherland, 1992; Ueberschär, 1995). This metabolic rhythm is linked to the environmental changes such as illumination, temperature, or food availability. For example, marine fish larvae have active feed intake and resting periods (or digestion stages) periodically under natural conditions. There are clear indications from field trials that circadian rhythm in the proteolytic digestive enzyme

capacity is closely linked to the natural light regime and, feeding activity occurs in many species specifically at sunrise and sunset (Kane, 1984; Ueberschär, 1995). Nevertheless, the predominant method in intensive larvae cultures is based on continuous illumination and frequent introduction of food. Under such conditions the larvae will capture prey continuously. For instance, gilthead sea bream larvae feed continuously without apparent satiation while the food is available (Rønnestad et al., 2013). However, due to a lack of a morphological and functional stomach in most marine fish larvae (Govoni et al., 1986), the digestive enzyme capacity is limited and it is still questionable, if continuous food supply is beneficial for growth and survival or if such conditions rather overstrain the digestive capacity in the larval stages.

In order to investigate the digestive processes of fish larvae in relation to feeding activity, tryptic enzyme activity was found to be an appropriate indicator (Lauff and Hofer, 1984; Pedersen et al., 1990; Ueberschär, 1995), specifically on a short term scale. For example, Drossou et al. (2006) determined the effects of two test diets on tryptic activity during early stages of Nile tilapia (*Oreochromis niloticus*). Tillner et al. (2013a) evaluated the daily pattern of tryptic enzyme activity of Atlantic cod larvae (*Gadus morhua*) under 1 meal or 2 meal feeding protocol, and more recently, Navarro-Guillen et al. (2015) examined diel food intake and tryptic enzyme activity of Sole Senegalese larvae under laboratory conditions. These studies have investigated the mealtime, gut fullness, prey density under different light conditions on digestion (Fujii et al., 2007; MacKenzie et al., 1999; Navarro-Guillen et al., 2015; Tillner et al., 2013a), but have not focused on the diurnal pattern of digestive enzymes under different feeding regimes.

Although a considerable improvement in the quality of formulated diets was achieved in the last decade (Cahu et al., 2003; Hamre et al., 2013), current feeding protocols of marine fish larvae are still depending mainly on live prey, specifically in the first-feeding stages and beyond in the early larval stages (Conceição et al., 2010b; Drossou et al., 2006; Holt et al., 2011). Several studies have been evaluating the feeding behavior and digestive physiology of sea bream larvae in regards to MicroDiet ingestion (Fernandezdiaz et al., 1994; Kolkovski et al., 1997a; Parra and Yúfera, 2000). Nevertheless, there are few and not conclusive data available to determine the optimum feeding time and frequency for the different developmental stages of the species specifically under a feeding regimes applying MicroDiets.

The aim of this study was to evaluate the impact of different dietary treatments on the diurnal rhythm of the digestive tryptic enzyme activity in gilthead sea bream (Sparus aurata) larvae and

to compare specifically among live and formulated feed. Therefore, several short-term experiments were conducted at different ages with different dietary treatments such as traditionally used live feeds (rotifers and Artemia) and MicroDiet.

2. Materials and Methods

2.1. Larval rearing

Larvae were obtained (3 days post hatch, dph) from the fish farm Valle Ca' Zuliani in Italy[1] and transported to the experimental facility of the GMA - Gesellschaft für Marine Aquakultur mbH Büsum[2], Germany. Larvae were reared in 65 L green conical tanks with a stocking density of 75 larvae L^{-1}. Light intensity was kept at 250 lux at the water surface via illumination above the tanks for 24 h. Oxygen was kept between 7.18 and 8.26 mg L^{-1} throughout the experiment and the water temperature was gradually increased (0.5°C/day) from 16.5 °C to 20.2 °C. pH was measured every day (7.84±0.43). Salinity was slowly decreased from 37 to 29 psu (1 psu/2 days between 3 dph to 15 dph) and kept till 31 dph, and from 32 to 44 dph kept at 28 psu. The seawater was filtered (polyester filter cartridges cascade 50, 20 and 5µm) and treated with UV light (55 Watt UVC), and circulated among the big tank and the 12 experimental tanks (Fig.I-1). A surface skimmer was installed in each small tank to remove constantly oil-like films on the water surface in order to facilitate swim bladder inflation. A protein skimmer was installed to remove organic debris from the big tank. Additionally, the water surface in the rearing tanks was skimmed daily with paper towel and accumulations of feed on the tank bottoms were frequently siphoned. All rearing tanks were equipped with a water inlet below the water surface and aerated with an air tube with gentle bubbles from the center of the bottom. Green water technique was applied and each tank received microalgae *Nannochloropsis sp*. paste (BlueBioTech, Germany) pre-mixed with seawater three times a day at 07:00, 14.00 and 22:00, respectively (2 mL, 3–23 dph; 1 mL, 23–26 dph, 800000 cells/ml).

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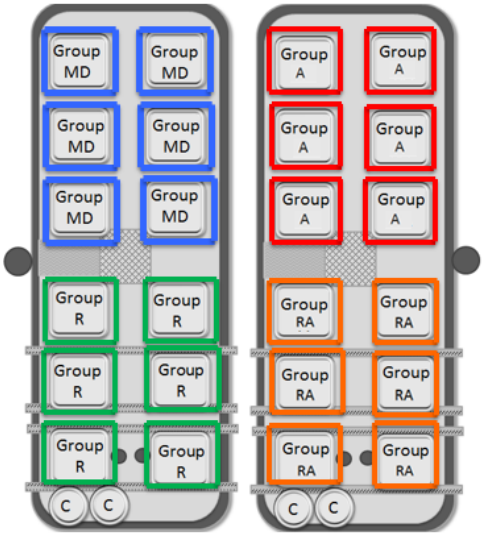

Figure I-1. Schematics of the experimental setup.

2.2. Experimental design and sampling

A 20 h experiment was conducted at four different ages with four different feeding regimes. Group R; sampled at 21, 26 and 34 dph, fed with rotifers (*Brachionus plicatilis*) at densities of 4 ml^{-1} , 6 ml^{-1}, 15 ml^{-1} respectively. Group RA; sampled at 26 dph, fed with rotifers + Artemia nauplii (INVE, Ghent, Belgium) at densities of 6 rotifers ml^{-1} and 1.3 Artemia ml^{-1} respectively. Group A; sampled at 44 dph, fed with Artemia nauplii at densities of 4 ml^{-1}. Groups fed with live feed fed three times a day at 07:15, 14:15 and 22:15 o'clock. Group MD, sampled at 26 and 44 dph, fed with Microdiet (Skretting, Belgium) every 15 minutes with an automatic feeder at about 30 mg diet per feeding event and per experimental tank. The control groups of about 300 larvae were randomly collected from the respective rearing tanks before the first feeding of that day and were transferred to separate tubes with clean seawater, and kept for gut evacuation (considered as the control group, group C). The cylindrical transparent plexiglass tubes (Ø 15cm) were placed inside the large tank and an extra air dispenser was installed for gentle aeration. An appropriate size of gauze was fixed on the bottom of the tubes for the larvae not to escape (and to prevent feed penetrating into the cylinder during the experiment), but also having water exchange during the experiment. Six tanks were assigned to each of the following groups (R, RA, A and MD) (see in Fig.I-1). Larva from each replicate tank (total n=6) representing each dietary treatment were collected every full hour and transferred into an Eppendorf vial between 06:30 - 01:30 o`clock and immediately stored at -80°C until analysis in order to stop any digestion process in the gut of larvae.

2.3. Sample preparation and analyses of tryptic enzyme activity

Each batch of frozen larvae from one sampling event (6 larvae) was placed on a petri dish stored on crushed ice to thaw slowly and following thawing rinsed with distilled water. Their total length (mm, from tip of snout to the posterior margin of body) was measured under microscope using a millimetric scale (Fig.I-2). Each individual was transferred into a 1.5 ml Eppendorf vial and homogenized in ice-cold Tris-Buffer (TRIS–HCl, 0.1M, pH 8, MERCK, Art.no. 1083820500, including $CaCl_2 \times 2H_2O$, 0.02M, MERCK, Art.no. 2382) by using a motorized pestle with variable amount of buffer, depending on the size, in order to maximize the activity signal in the measurement procedure (21, 26 and 34 dph larvae with 125µL or 250µL; 44 dph larvae with 500 µL). The homogenate was centrifuged for 45 min (4110xg at 0°C) to settle the tissue fragments; subsequently the supernatant was used for the analysis of tryptic enzyme activity. The tryptic enzyme activity of each individual larva sample was analyzed using a fluorescence technique according to Ueberschär (1993). The synthetic fluorescence substrate used was Nα-benzoyl-L-arginine-methyl-coumarinyl-7-amide-HCl (BACHEM, Art.No: 4002540.0250). All samples were kept on ice during the preparation of individual larval samples for trypsin enzyme activity measurement in order to avoid any proteolytic activity. Tryptic activity is expressed as the amount of hydrolysed substrate (µmol MCA) per minute and larva.

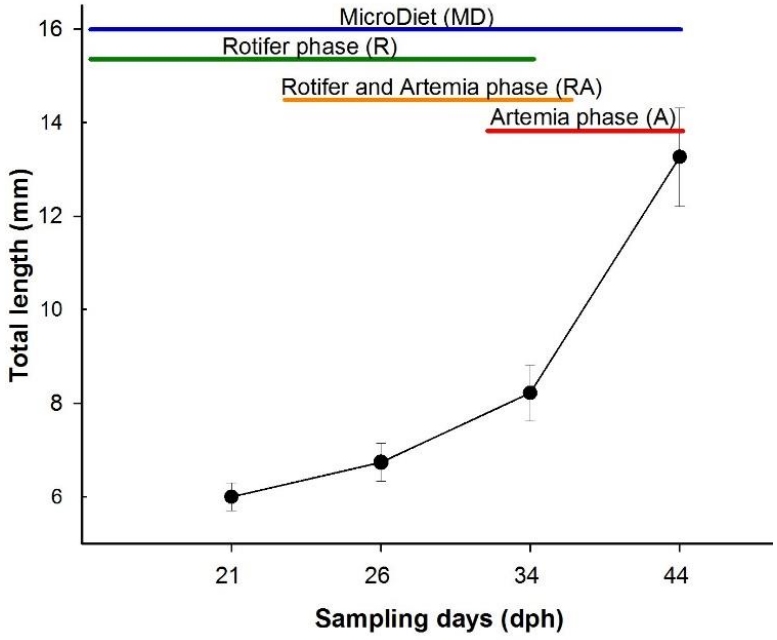

Figure I-2. Feeding regime of different groups and total length of sampled sea bream larvae over the experimental period of 44 days. Data are presented as mean ± S.D (n=120 at 21 dph, n=360 at 26 dph, n=120 at 34 dph, n=240 at 44 dph).

2.4. Statistical analyses

The statistical software R (2014) was applied to evaluate the data. The data evaluation started with the definition of an appropriate statistical model based on generalized least squares (Carroll and Ruppert, 1988; Davidian and Giltinan, 1995). The data were assumed to be normally distributed and to be heteroscedastic due to the different levels of feeding groups, age and sampling event. These assumptions are based on a graphical residual analysis. The statistical cell means model (Schaarschmidt and Vaas, 2009) included a pseudo factor representing the combination of the actual factors feeding groups (group R, RA, A and MD), age (21, 26, 34 and 44 dph) and sampling event (from 06:30 to 01:30). This was necessary because the experiment did not include all combinations of feeding groups, age and sampling event. Based on this model, multiple contrast tests for heteroscedastic data according to Hasler and Hothorn (2008) were conducted in order to compare the several levels of the influence factors, respectively (R package SimComp, 2014). The results are given as mean and differences were considered statistically significant at $p<0.05$.

3. Results

3.1. Larval length

There were no significant differences in the individual lengths of the larvae, neither between the control groups and the fed larvae nor between different feeding regimes at the sampling events at the same age throughout the entire experiment (mult comp test, $p< 0.05$).

3.2. Groups fed with rotifers

Three different age groups (21, 26, and 34 dph) of sea bream larvae were fed on rotifers according to the feeding protocol. The comparison of tryptic enzyme activity of fed and control group over 20 h are shown in Fig. I-3. The mean tryptic activity (nmol MCA min^{-1} $larva^{-1}$) ranged between 1.064±0.8 at 21 dph, 1.025±0.41 at 26 dph and 1.417±0.48 at 34 dph and there were no significant differences among fed 21, 26, 34 dph larvae groups. There was a significant difference in lengths between 21 dph and 34 dph of larvae (data not shown). The tryptic enzyme

activity did not show any significant differences among fed 21, 26, and 34 dph age larvae groups (mult comp test, $p< 0.05$).

At 21 dph (Fig. I-3A) the diurnal pattern of tryptic enzyme activity showed various peaks and drops between each sampling event. 1 h after second feeding, the tryptic enzyme activity depicts steep increase to 4.04 nmol MCA min^{-1} $larva^{-1}$ and peaked at 15:30. Furthermore, tryptic enzyme activity remained at the lowest level between 18:30 and 21:30. At 26 dph (Fig. I-3B) the diurnal pattern of tryptic enzyme activity was similar to 21 dph larvae group; however the fluctuation in tryptic enzyme activity with peaks and drops remained constant over 2 h. The highest tryptic enzyme activity was 1.92 nmol MCA min^{-1} $larva^{-1}$ at 16:30, 2 h after second feeding event. At 34 dph (Fig. I-3C) a clear difference between fed and control larvae can be observed regarding the enzyme activity pattern throughout the sampling period of 20 h.

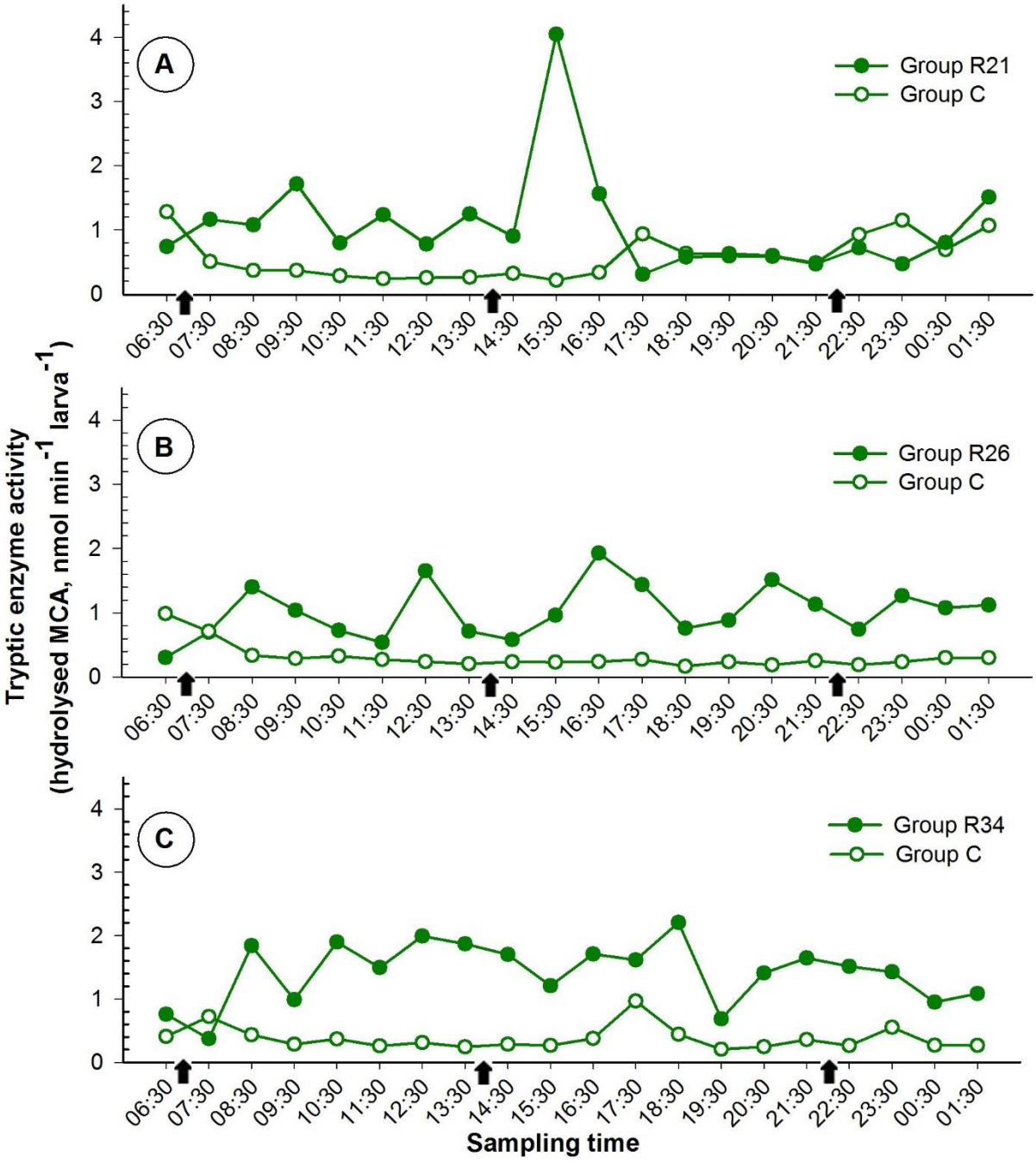

Figure I-3. Diurnal pattern of tryptic enzyme activity (nmol MCA min^{-1} $larva^{-1}$) levels of fed (●—) and control (—○—) sea bream larvae at three different age groups (A) 21 dph, (B) 26 dph, and (C) 34 dph. Sampling of fed and control larvae took place between 06:30 to 01:30 every full hour under 24 h of light. Data are presented as mean (n=6/sampling time). Black arrows indicate the administration of rotifers three times a day. No symbols indicate no significant differences between control and fed groups (mult comp test, $p< 0.05$).

3.3. Group fed with rotifer and Artemia

Results for the tryptic enzyme activity of 26 dph sea bream larvae fed with rotifer and Artemia are depicted in Fig.I-4A. Fed sea bream larvae showed a clear pattern and diurnal variation of tryptic enzyme activity and an immediate response after the feeding events. The highest tryptic activity was found at 14:30, and the lowest activity was observed from 20:30 to 22:30. The results showed that tryptic activity in the fed group increased gradually 2 h after the first administration of rotifers and Artemia from 0.52 ± 0.30 to 7.95 ± 0.03. In the fed group following the second administration of rotifers and Artemia the tryptic enzyme activity reached immediately the maximum level of 9.25 ± 1.67.

3.4. Group fed with Artemia

At 44 dph (Fig. I-4B) the tryptic enzyme activity pattern showed a distinct increase after each feeding event. The highest activities occurred 3 h after feeding events at 10:30 (125.25±53.32), at 18:30 (115.24±115.99) and at 01:30 (106.69±53.51). The lowest level of tryptic activity were observed at 13:30, 14:30 and 22:30 where significant differences in activity was detected in relation to other sampling events (mult comp test, $p<0.05$).

3.5. Groups fed with MicroDiet

Two different age groups (26 and 44 dph) of sea bream larvae were continuously fed with MicroDiets according to the feeding protocol. The mean tryptic activity ranged between 4.74±4.40 at 26 dph and 56.40±31.55 at 44 dph (Fig. I-4A and I-4B). There was a significant difference in lengths between 26 dph and 44 dph of larvae, however the mean tryptic enzyme activity showed significant differences only at 12:30, 18.30 and 20:30 (data not shown) (mult comp test, $p<0.05$).

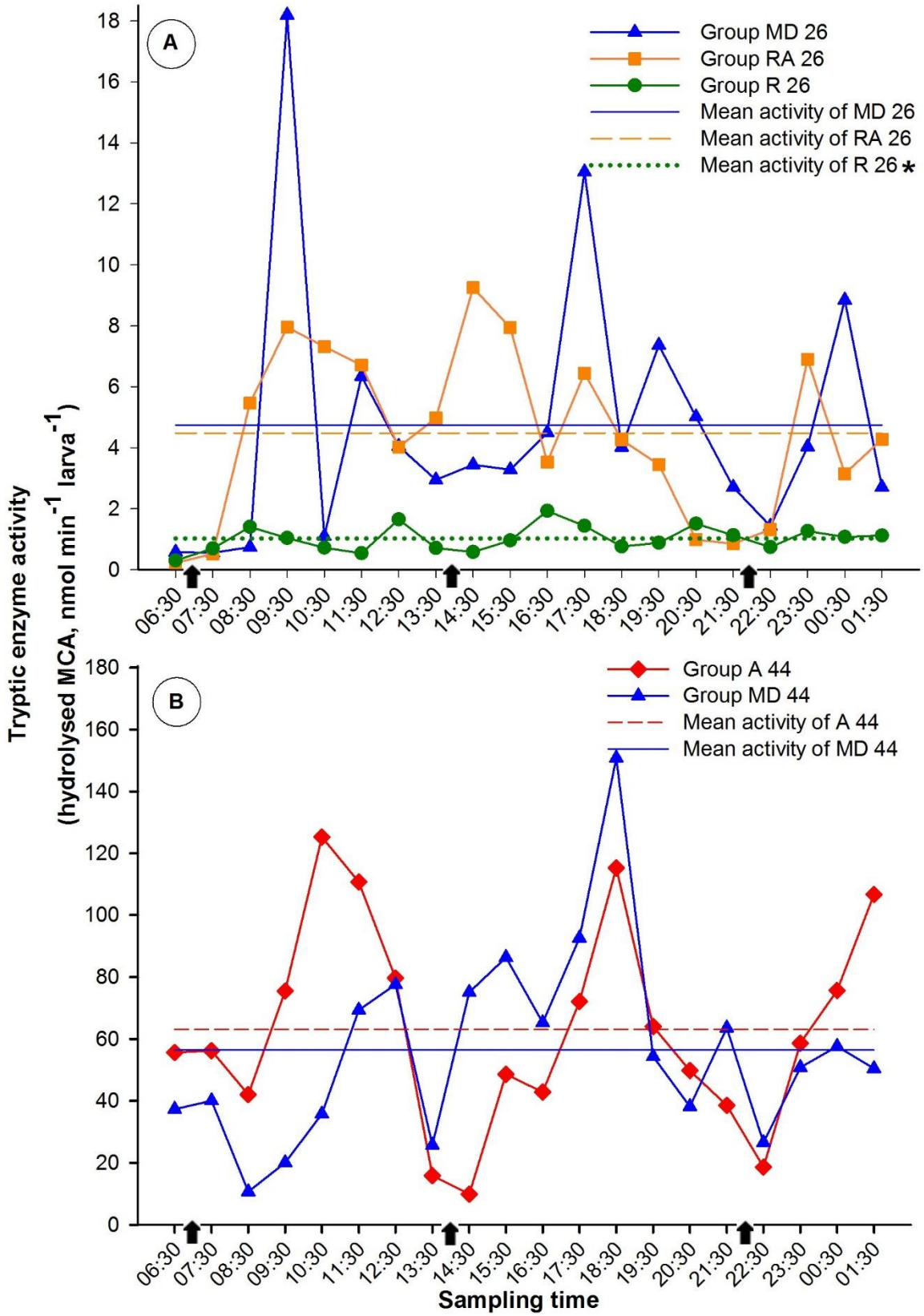
A
Group MD 26
Group RA 26
Group R 26
Mean activity of MD 26
Mean activity of RA 26
Mean activity of R 26 *
B
Group A 44
Group MD 44
Mean activity of A 44
Mean activity of MD 44
Tryptic enzyme activity
(hydrolysed MCA, nmol min-1 larva-1)
Sampling time
06:30
07:30
08:30
09:30
10:30
11:30
12:30
13:30
14:30
15:30
16:30
17:30
18:30
19:30
20:30
21:30
22:30
23:30
00:30
01:30

Figure I-4. (A) Diurnal pattern of tryptic enzyme activity (nmol MCA min^{-1} larva-1) of rotifer fed; group R (—○—), rotifer and Artemia fed; group RA (—■—) and MicroDiet fed; group MD (—▲—) sea bream larvae at 26 dph. (B) Diurnal pattern of tryptic enzyme activity (nmol MCA min^{-1} $larva^{-1}$) of Artemia fed; group A (—◈—) and MicroDiet fed; group MD (—▲—) sea bream larvae at 44 dph. Sampling of each group took place between 06:30 to 01:30 every full hour under 24 h of light. Data are presented as mean (n=6/sampling time). Dotted lines indicate the mean tryptic enzyme activity of group R26, dashed lines indicate the mean tryptic enzyme activity of group RA26 and group A44, and straight lines indicate group MD26 and MD44 respectively. Black arrows indicate the administration of rotifers and Artemia three times a day, where MicroDiet group was fed every 15 minutes. Asterisk indicate significant differences between group R26 and; group RA 26 and group MD 26 overall the experiment. No symbols indicate no significant differences (mult comp test, $p< 0.05$).

4. Discussion

The effect of different dietary treatments, such as traditionally used live feeds (rotifers and Artemia) and MicroDiet, at different ages on the diurnal tryptic enzyme activity in sea bream larvae was evaluated. Based on an hourly monitoring of tryptic enzyme activity over 20 h, it can be concluded that a diurnal pattern in tryptic enzyme activity exists in sea bream larvae at different ages in the groups fed with Artemia and MicroDiets, but less pronounced in the early stages fed with rotifers only. Concluding, the various patterns are obviously linked to the kind of feeding regime.

Among the different feeding regimes, there were noticeable differences in tryptic enzyme activity. In general, the tryptic enzyme activity was significantly lower in groups fed with rotifers (group R21, R26 and R34, Fig.I-3A,B,C) compared with groups fed with Artemia and MicroDiet (group RA26, MD26, A44 and MD44, Fig.I-4A,B). This result is in line with the study by Gamboa-Delgado et al. (2011) demonstrating that trypsin activity in Senegalese sole larvae fed with rotifers showed lower activity compared to Artemia and larvae fed with formulated diets respectively. In addition, in our experiments the groups fed with rotifers had lower gut fullness (between 25 to 50%) compared to Artemia and MicroDiet groups (between 75 to 100%) (data not shown). Gut fullness and the interaction of feed-gut (mechanical stimulation, osmotic

conditions, etc.) could have an effect on trypsin secretion as well. The mechanical stimulation in the gut is different (more “bulky” food particles causes probably a higher stimulation) including a different degree of gut fullness according to the type of food (with Artemia and MicroDiets, the larvae might tend to ingest more particles in a short period compared to e.g. rotifers). This could be the reason of the small fluctuations in group R whereas the more pronounce peaks after meal time in group RA and A. These differences in the pattern of tryptic activity might be justified with the different feeding behavior of larvae at different feeding regimes. When feeding an organism which is easy to digest, the larvae have to invest less enzyme capacity and can accomplish a rather "economic mode" in digesting the food and/or trigger substances from the food causes a different reaction in trypsinogen secretion. Recent tube-feeding experiments showed that some natural products which contain high level of protein concentration had a positive trigger effect on the tryptic activity in sea bass larvae (Zeytin et al., in preparation). Moreover, a third meal of rotifers and Artemia did not result in tryptic enzyme activities as high as after the first and second feeding events, suggesting a limited proteolytic capacity in sea bream larvae and prevents to benefit of a continuous supply of feed in a short time period. These finding is line with Tillner et al. (2014) where they pointed that a reduction in tryptic enzyme activity after several feeding events indicates a limit in diurnal digestive capacity in sea bass larvae. The degree to which fish larvae are able to accommodate feed in the gut and to digest feed efficiently might be depending on species, age (Lauff and Hofer, 1984) and feeding regime (Kolkovski, 2001). Thus, the present results suggest that the third meal could be considered as nonessential, at least in sea bream larvae in the developmental stages as investigated in this study. Although, the reaction of the third meal is rather weak compared to the previous feeding events, the mean tryptic enzyme activity value among fed and control group throughout the experimental period was significantly different (mult comp test, $p<0.05$). This low activity value in the control group without showing peaks and drops demonstrates the significance of a mechanical stimulation of the digestive tract as consequences of the ingestion of feed.

The comparison of the tryptic activity in two different fed groups (group A and MD) at 44 dph showed a quite similar diurnal pattern in tryptic enzyme activity although group MD has been continuously fed with MicroDiet (Fig. I-4B). The mean tryptic enzyme activity of group A and MD (as mean value with 95% confidence limit) was very similar with no significant differences, suggesting that both groups do "invest" the same amount of trypsinogen to digest the different

diets. In contrast to the feeding groups, tryptic enzyme activity remained significantly lower in group C with larvae deprived of food throughout the entire sampling period which confirms the significance of feeding and uptake of food as a physical and biochemical trigger, provoking trypsinogen secretion into the gut. Based on the size measurements over the entire period of 45 days, there were no significant differences in terms of TL at same age under different feeding regimes (between R26, RA26, MD26 and A44, MD44).

In summary, this study demonstrates that a limited digestive capacity in sea bream larvae exists at some point in time during the day, no matter what kind of diet was applied. It is suggested, that early larval stages might not be able to digest the applied feed over 24 h with constant efficiency. In general, these results should be considered for the application of suitable feeding schedules with a proper quantity in the early stages of marine fish larvae to maximize nutrient utilization and growth, and, in case of MicroDiet feeding, to minimize detrimental effects on the water quality due to the oversized feeding events.

Based on the results in this study, further helpful conclusions in relation to an optimization of feeding protocols could come from experiments, where feeding is being reduced in the afternoon and evening respectively and compared with continuous food supply under 24 h illumination. To get the complete picture on the variability of the major enzymes participating in early larval stages other enzymes, such as pepsin, amylase, and lipases should be investigated in addition to trypsin.

Acknowledgments

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CHAPTER II

The potential of various triggering substances to enhance the tryptic enzyme activity in early stages of sea bass (*Dicentrarchus labrax*) larvae

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Abstract

Many marine fish larvae show deficiencies of tryptic enzyme activity in early stages which limits the digestive process and utilisation of feeds efficiently from first-feeding. Since first feeding with microparticulated feed becomes now reality, there are options to add ingredients to artificial feed which can improve digestive capacity. Therefore, the present study aimed to identify substances that can enhance the tryptic enzyme activity in various age stages of sea bass larvae. We have investigated the short-term effects (incubation time of 0.5, 1, 2, 4, 8 and 10 hours) of specific ingredients via tube-feeding method. As a model study, six different substances with different concentrations were tested in larvae between 30 to 60 dph, which may also apply to the earlies stages. Larvae tube-fed with physiological saline solution applied as a control. Results revealed that tryptic enzyme activity was higher compared to the control groups when applying phytohaemagglutinin, protein concentrate, Calanus meal and the extract of the commercial microdiet MiniPro with some distinct differences among age and concentrations. Tryptic enzyme activity in larvae was generally lower in bovine serum albumin and spermine compared to other tube-fed trigger substances. However, it was also demonstrated that the injected substances, their concentration and incubation time on different age have a significant impact on the amount of trypsinogen secretion in the gut of sea bass larvae. In summary, the present study provides a useful matrix, which substance may be of advantage in which concentration and at which life stage of marine fish larvae.

Key words: Digestive capacity; *Dicentrarchus labrax*; Sea bass larvae; Triger substances; Trypsin; Tube-feeding

1. Introduction

Considerable amount of research effort has been invested in the development of formulated diets that can either partially or completely replace live feed at least during certain larvae stages (Andrés et al., 2011; Baskerville-Bridges and Kling, 2000b; Cahu and Zambonino Infante, 2001; Cahu et al., 1998; Fletcher et al., 2007; Gallardo et al., 2013; Sarvi et al., 2010; Yúfera et al., 2000). Nevertheless, precise knowledge on fish larval nutritional requirements, their digestive capacities and implications for the formulation of microdiets are still limited for many fish species during the larval period (Blair et al., 2002; Cahu and Infante, 1997; Hamre et al., 2013;

Rønnestad et al., 2013; Tillner et al., 2013b). The challenging "critical" period in which marine fish larvae show deficiencies in tryptic enzyme capacity, are among the major bottlenecks related to the complete replacement of live feed by microdiets (Hamre et al., 2013; Kolkovski, 2001; Ueberschär, 2006; Yúfera et al., 2000). In combination with the formulation of microdiets for fish larvae, it is of interest to examine how deficiencies in larval digestion capacity can be manipulated by the addition of nutrients to overcome the lack of proteolytic capacity by facilitating the production and secretion of trypsinogen. This could result in an improvement of digestive capacity and promote eventually the survival rate during the early larval stages specifically in marine species.

In order to raise larval digestive enzyme capacity and digestion efficiency, few studies have tested the effect and larval response of specific nutrients in artificial diets for marine fish larvae (Cahu et al., 2004; Gisbert et al., 2012; Kotzamanis et al., 2007; Tovar et al., 2002). Although the results of tracer studies demonstrated that hydrolysed proteins are absorbed and processed faster than complex proteins (Conceição et al., 2011; Conceição et al., 2007; Rønnestad et al., 2007), there are still controversial discussions about whether pure or hydrolysed proteins are more effective in microdiets (Hamre et al., 2001; Kvåle et al., 2009; Savoie et al., 2006; Tonheim et al., 2005; Zambonino Infante and Cahu, 2001).

In previous studies, it has been found that the administration of different protein sources as substances in diets for fish larvae had a direct impact on the digestive enzyme profile. Such as bovine serum albumin (BSA) (as almost pure protein source) in Atlantic herring larvae (*Clupea harengus*) (Koven et al., 2002); phytohaemagglutinin (PHA) (a plant derived complex protein) in Nile tilapia (*Oreochromis niloticus*) (Drossou et al., 2006) and sea bass larvae (*Dicentrarchus labrax*) (Tillner et al., 2014); spermine (as a natural polyamine) in sea bass larvae (Péres et al., 1997); products from copepods (as complex mixture of nutrients) were also being considered as a potential ingredient for artificial diet (Colombo-Hixson et al., 2012).

Due to the sensitive nature of larvae, time-age dependent measurements in the same individual larvae within a study on functional digestive physiology are difficult. Hence, the tube feeding method is an appropriate measure to evaluate the physiological response and effectiveness of potential trigger substances (Rønnestad et al., 2001) as applied in this study.

The present study aimed to identify substances that can enhance the trypsinogen production and secretion in various age stages of sea bass larvae. The tested substances were selected according

to previous results from studies which focused on some of the substances as potential ingredients to be used in the formulation of microdiets for marine fish larvae.

We specifically investigated the short-term effects (incubation time of 0.5, 1, 2, 4, 8 and 10 hours) of substances -BSA, PHA, protein concentrate, spermine, Calanus meal- with lower and upper limits of concentrations on the proteolytic capacity of different stages of sea bass larvae. In addition, an extract of a commercial microdiet (MiniPro) was also tested to evaluate the effect of a complete diet for fish larvae under tube-feeding conditions and in comparison, to individual substances. This study is supposed to provide a comprehensive comparison of a number of different substances at the same time in certain stages of sea bass larvae. The aim is, to provide a matrix, which substance may be of advantage in which concentration and at which life stage of marine fish larvae.

2. Materials and Methods

2.1. Larval rearing

Sea bass larvae (5 days post hatch, dph) were obtained from a commercial hatchery in Gravelines, France[3] and transported to the experimental facility of the GMA[4], Germany. The larvae were equally distributed to 12 green, conical tanks (65 L water volume each) with a stocking density of 150 larvae L^{-1} and kept in darkness until yolk sac absorption and mouth opening at 9 dph (French technique e.g. Moretti (1999). Larval rearing was carried out in a semi-closed sea water system as described by Zeytin et al. (2016) (see Fig.II-1). Oxygen was kept at 7.9±0.5 mg/l throughout the experiment and water temperature was gradually increased from 14 °C to 18 °C. Light regime was 16 h light/8 h darkness (07:00 – 23:00) and light intensity was increased from 50 to 500 lux at the water surface during the experiment. pH was measured every day (7.9±0.2). Salinity was slowly decreased from 38 to 28 psu. A surface skimmer was installed in each small tank to remove constantly oil-like films on the water surface in order to facilitate swim bladder inflation of larvae. A protein skimmer was installed to remove organic debris from the tank water. Additionally, the water surface was skimmed with paper towel and accumulations of dead larvae and debris on the tank bottoms were siphoned daily. All experimental tanks were equipped with a water inlet below the water surface and aerated with an air tube with gentle

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bubbles from the center of the bottom. Green water technique (Lavens and Sorgeloos, 1996b) was applied and each small tank received 1 mL microalgae *Nannochloropsis* sp. concentrate (BlueBioTech, Germany, 600.000 cells/ml) pre-mixed with seawater two times a day at 09:00 a.m. and 06:00 p.m., respectively. Sea bass larvae were co-fed with rotifers (density of 2 rotifers/ml) (*Brachionus* sp.; Meerwasser Center Menzel, Germany) and newly hatched *Artemia* instar I nauplii (AF 430 *Artemia*, INVE, Belgium) (density of 3 *Artemia*/ml increased to 8 *Artemia*/ml) three times a day (09:00, 15:00, 18:00) from mouth opening on throughout the experiment. In addition, larvae were fed with enriched rotifers (S.Presso, INVE) from 12 dph to 25 dph. From 26 dph on, larvae were fed with enriched *Artemia* instar II nauplii (Sanders Premium Cysten enriched with S.Presso, INVE) until the end of the experiment.

2.2. Tube-feeding

To evaluate the physiological response of potential trigger substances, the tube-feeding method which was developed by Rust et al. (1993) and modified by Rønnestad et al. (2000) was chosen and applied *in vivo* in different age groups of larvae. Groups of ca 300 larvae were randomly collected from the rearing tanks before the first feeding of that day and were transferred to separate cylindrical transparent plexiglass tubes (Ø 15cm) which were placed inside the large tank with an extra air dispenser for gentle aeration. An appropriate size of gauze (300 µm) was fixed on the bottom of the tubes for the larvae not to escape (and to prevent feed penetrating into the cylinder during the experiment) but allowing water exchange during the experiment. The larvae were then fasted for at least 14 h to ensure that their guts were empty. Larvae were removed individually from the tubes with a wide opening pipette and anaesthetised in a Petri dish with 30 µg/ml MS-222 (Tricaine methanesulfonate, SIGMA-ALDRICH). Six different substances (origins from enzymes, hormones, plant, animal and marine protein) which have the potential to improve the proteolytic digestive capacity were tested, each in three different concentrations (lower-middle-higher) to verify the best range for triggering trypsinogen secretion in different age stages of sea bass larvae (see in Table.1). BSA was applied only at two different concentrations. Physiological saline solution containing 0.9% NaCl (B.BRAUN, Art.no. 951 1261) was applied as a control. Before tube-feeding, ten extra larvae were individually weighed on a micro-balance in order to adjust the concentrations of the experimental solutions according to the average wet weight. An injection volume of 27.6 nL was set for each substance.

Table II-1. Substances, their concentrations and incubation times used in tube-feeding experiments. (All substances were dissolved in physiological saline solution to get the final concentrations).

Substance	**Concentrations in the tube-fed solution**	**Incubation time (h)**	**Age (dph)**
BSA [1]	5µg, 15µg	0.5, 1, 2, 4	33, 40
Spermine [2]	0.10µg, 0.33µg, 0.66µg	2, 4, 8, 10	36, 45
Protein concentrate [3]	5µg, 15µg, 30µg	0.5, 1, 2, 4	37, 46
Calanus meal [5]	1µg, 3µg, 6µg	0.5, 1, 2, 4	38, 44, 60
MiniPro [6]	1µg, 3µg, 6µg	0.5, 1, 2, 4	39, 47, 60
PHA [7]	0.001%, 0.003%, 0.006% of wet weight	0.5, 1, 2, 4	31, 44, 59

[1] Albumin bovine Fraction V, (Bovine serum albumin), Product No. 11924, SERVA

[2] Spermine ≥ 97%, Product No. S3256, Sigma-Aldrich

[3] Salmigo® L. The Marine Peptides, Product No. 380. 90% Protein in dry matter. Based on Salmon Proteins

[5] Calanus meal, defatted. Calanus AS, N-9272 Tromsø, Norway.

[6] MiniPro, Sea garden AS, 5525 Haugesund, Norway

[7] Phytohemagglutinin, L8754, Sigma-Aldrich

Six larvae were tube-fed for each different substance and concentration with their control group and incubation period (total N=1392, see in Fig.II-1). After injection of the trigger substance, each larva was rinsed with clean sea water and the larvae were incubated in 6-well multidishes under the same conditions as in the rearing tank. The larvae endured the injection in good condition and were kept in the multidishes until sampling (0.5 to 10 hours). Incubation times were chosen according to Applebaum and Ronnestad (2004). Each larva was gently transferred to an Eppendorf tube and immediately preserved at -80 °C in order to stop any biochemical processes in the gut. The time taken by the tube-feeding operation was about 60 s, and the total transfer time was about 15 s. No larval mortality occurred as a result of tube-feeding.

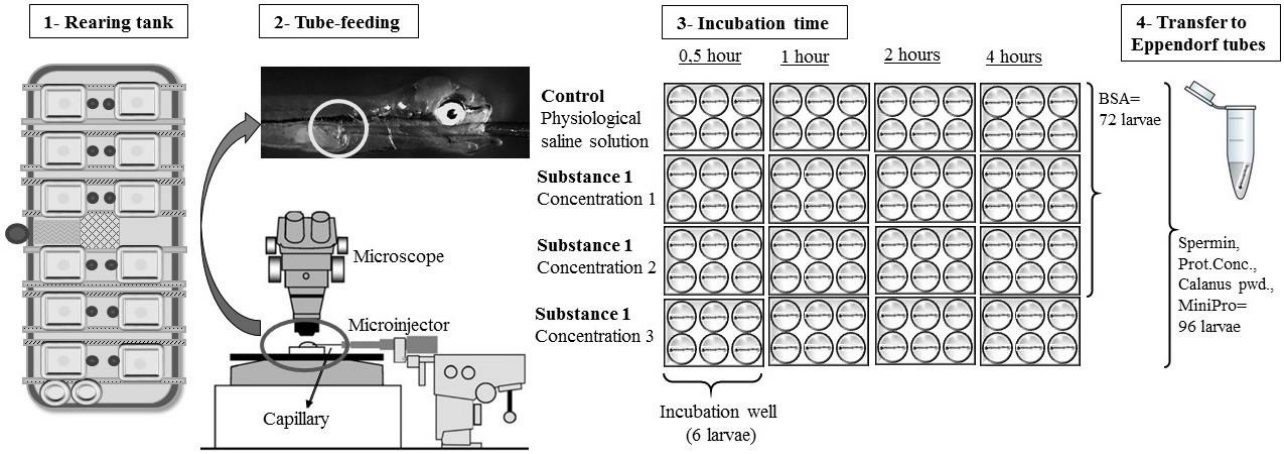

Figure II-1. 1-Rearing system for sea bass larvae throughout the experiment (Zeytin et al., 2016). 2-Set-up for tube-feeding of fish larvae via micro-injection (Rønnestad et al., 2001). Photo represents the amount of injected substance in sea bass larvae. During the tube-feeding experiment, larval gut was empty. 3-Design of tube feeding for each substance, concentration and incubation time throughout the experiment. Number of larvae presented for one age group. 4- Transferred of each tube-fed larva to separate Eppendorf tubes.

2.3. Sample preparation and analyses of tryptic enzyme activity

Each frozen larva was placed on a petri dish stored on crushed ice to thaw slowly and afterwards rinsed with distilled water. Their total length (mm, from tip of snout to the posterior margin of body) was measured under a microscope using a millimetric scale. Each individual was transferred into a 1.5 ml Eppendorf vial and homogenized in 250 µL ice-cold Tris-Buffer by using a motorized pestle. The homogenate was centrifuged for 45 min (4110 x g, 0 °C) to settle the tissue fragments. The supernatant was used for the analysis of tryptic enzyme activity.

The tryptic enzyme activity of each individual larva sample was analyzed using a fluorescence technique according to Ueberschär (1993). The synthetic fluorescence substrate was Nα-benzoyl-L-arginine-methyl-coumarinyl-7-amide-HCl (BACHEM, Art.No: 4002540.0250) and the buffer was TRIS–HCl (0.1M, pH 8) (MERCK, Art.no.1083820500) including CaCl2×2H2O (0.02M) (MERCK, Art.no. 2382). All samples were kept on ice during the preparation of individual larvae for trypsin assays to avoid any proteolytic activity prior to the measurements. Tryptic activity is expressed as hydrolysed fluorescent products MCA (methyl-coumarinyl-7-amide, nmol MCA min^{-1} $larva^{-1}$). A MCA standard curve was used to calculate the tryptic activity in the samples.

2.4. Statistical analyses

The statistical software R (2017) was used to evaluate the data. The data were assumed to be normally distributed and to be heteroscedastic. These assumptions are based on a graphical residual analysis. The statistical model of the data is based on a pseudo factor, which represents a mixture of the actual factors: substance, concentration, incubation time, and age (Schaarschmidt and Vaas, 2009). Such a pseudo one-way model is necessary here because the data set is not orthogonal. Based on this model, multiple contrast tests for heteroscedastic data according to Hasler and Hothorn (2008) (R package SimComp, 2014) were conducted in order to compare the several levels of the actual influence factors. The resulting multiplicity adjustment was a mixture of a multivariate and a Bonferroni adjustment, taking into account all influence factors to provide a robust and careful statistical evaluation of the data. Thus, only the most robust and significant physiological effects were considered in this work. The results are given as mean ± SD. Differences were considered statistically significant at $p<0.05$.

All possible combination of factors that could have an effect on tryptic enzyme activity were statistically compared. To understand what are the limiting and influencing factors for digestive physiology, we particularly conducted the following tests: (T1) We tested all influence factors (trigger substance, incubation time, age and concentration, 232 groups) against the overall mean of all groups to find out which groups are significantly better than the average. (T2) For all combinations (60 groups) of substance, incubation time and age, we did comparisons of the several concentrations against the control. (T3) For all combinations (43 groups) of substance, age and concentration, we did all-pair comparisons of the several incubation times. (T4) For all combinations (68 groups) of substance, incubation time, and concentration, we did all-pair comparisons of the several ages (see in Table. 2).

3. Results

Tube-fed larvae were not significantly different in standard length (i.e. the ontogenetic stage was the same) between different groups at all sampling points (data not shown). A summary of all significant results is shown in Table.2.

The amount and pattern of tryptic enzyme activity was different for each tube-fed trigger substance, concentration and age group in sea bass larvae. The results of all administered trigger substances are presented in Fig. II-2 - 6. Tryptic enzyme activity (nmol MCA min^{-1} $larva^{-1}$) was higher than the control groups when applying PHA, protein concentrate, Calanus meal, and the extract of MiniPro with some distinct differences among different age and concentrations in tube fed sea bass larvae. However, only the injected 1µg of extracted MiniPro showed a significantly higher tryptic activity (4.16±0.24 nmol MCA min^{-1} $larva^{-1}$) at 60 dph after 4 h of incubation (Fig.II-2) (mult comp test, $p< 0.05$). In addition, the extract of the commercial microdiet MiniPro showed the highest activity (6.41±1.50 nmol MCA min^{-1} $larva^{-1}$) after 4 h of incubation at 60 dph.

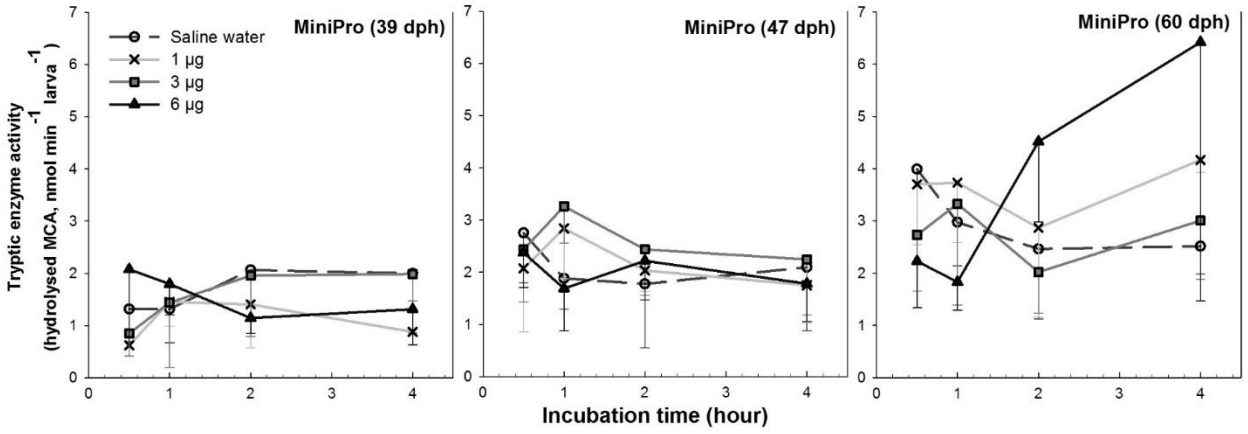

Figure II-2. Age and time response of tryptic enzyme activity (nmol MCA min^{-1} $larva^{-1}$) levels of tube-fed sea bass larvae under different concentration of MiniPro and control at 39, 47 and 60 dph. Values are given as mean (n=6/incubation time) ± SD.

Tryptic activity increased significantly in larvae treated with the lowest concentration (0.001%) of plant protein phytohemagglutinin (PHA) at 59 dph. On the other hand, the highest level observed at 4 h after injection (4.96±1.16 nmol MCA min^{-1} $larva^{-1}$). In contrast, the highest concentration (0.006%) showed decreased in tryptic enzyme activity in all age groups (Fig.II-3).

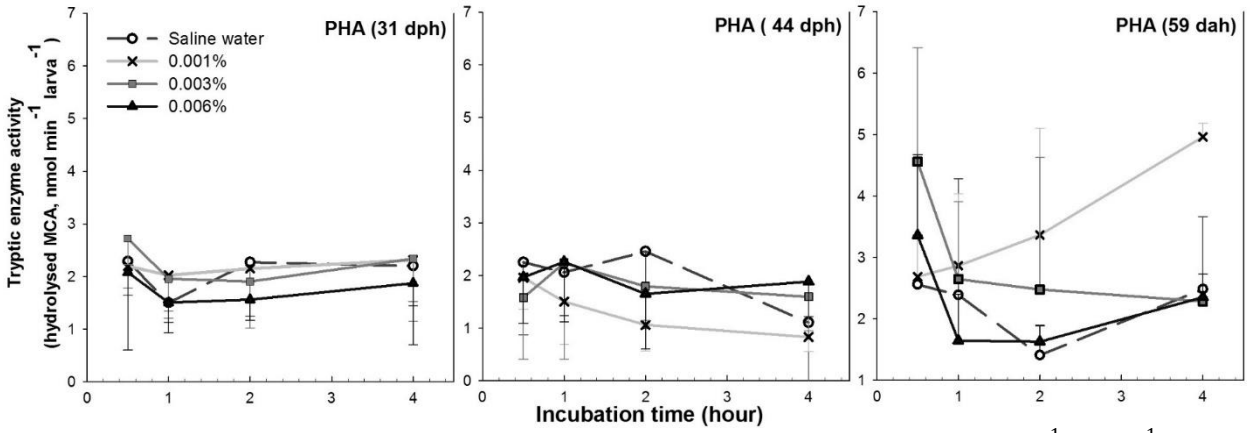

Figure II-3. Age and time response of tryptic enzyme activity (nmol MCA min^{-1} $larva^{-1}$) levels of tube-fed sea bass larvae under different concentration of PHA and control at 31, 44 and 59 dph. Values are given as mean (n=6/incubation time) ± SD.

Calanus meal with 3µg concentration showed the most significant trypsin activity (4.40±0.46 nmol MCA min^{-1} $larva^{-1}$) after 4 h of incubation at 60 dph (mult comp test, $p< 0.05$) (Fig.II-4).

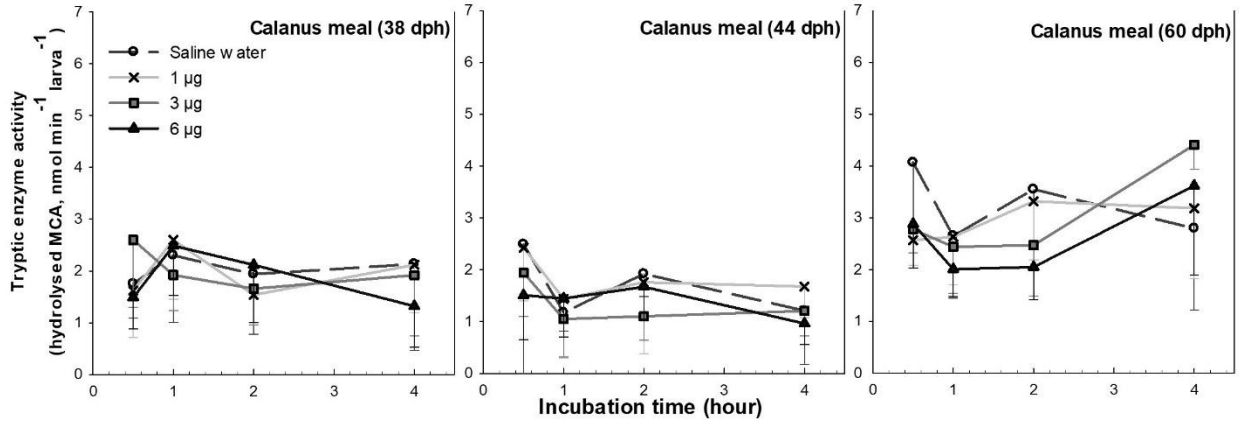

Figure II-4. Age and time response of tryptic enzyme activity (nmol MCA min^{-1} $larva^{-1}$) levels of tube-fed sea bass larvae under different concentration of Calanus meal and control at 38, 44 and 60 dph. Values are given as mean (n=6/incubation time) ± SD.

Protein concentrate (Salmigo® L) at lowest concentration (5 µg) showed positive effect and higher trypsin activities in both age groups (37 and 46 dph). However, the activities were not significantly different from the control and other concentrations (Fig.II-5).

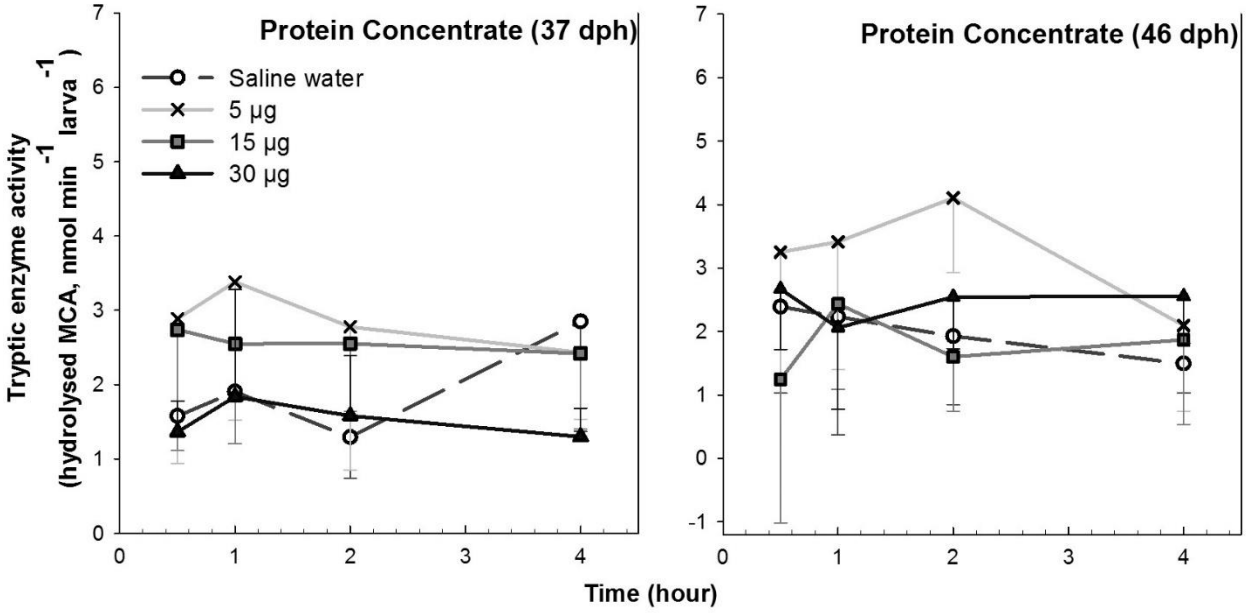

Figure II-5. Age and time response of tryptic enzyme activity (nmol MCA min^{-1} $larva^{-1}$) levels of tube-fed sea bass larvae under different concentration of protein concentrate and control at 37 and 46 dph. Values are given as mean (n=6/incubation time) ± SD.

Tryptic enzyme activity in larvae was generally lower in larvae fed with BSA and spermine compared to other tube-fed trigger substances and showed in average of 2.11±0.97 and 1.53±0.74 nmol MCA min^{-1} $larva^{-1}$, respectively (Fig.II-6).

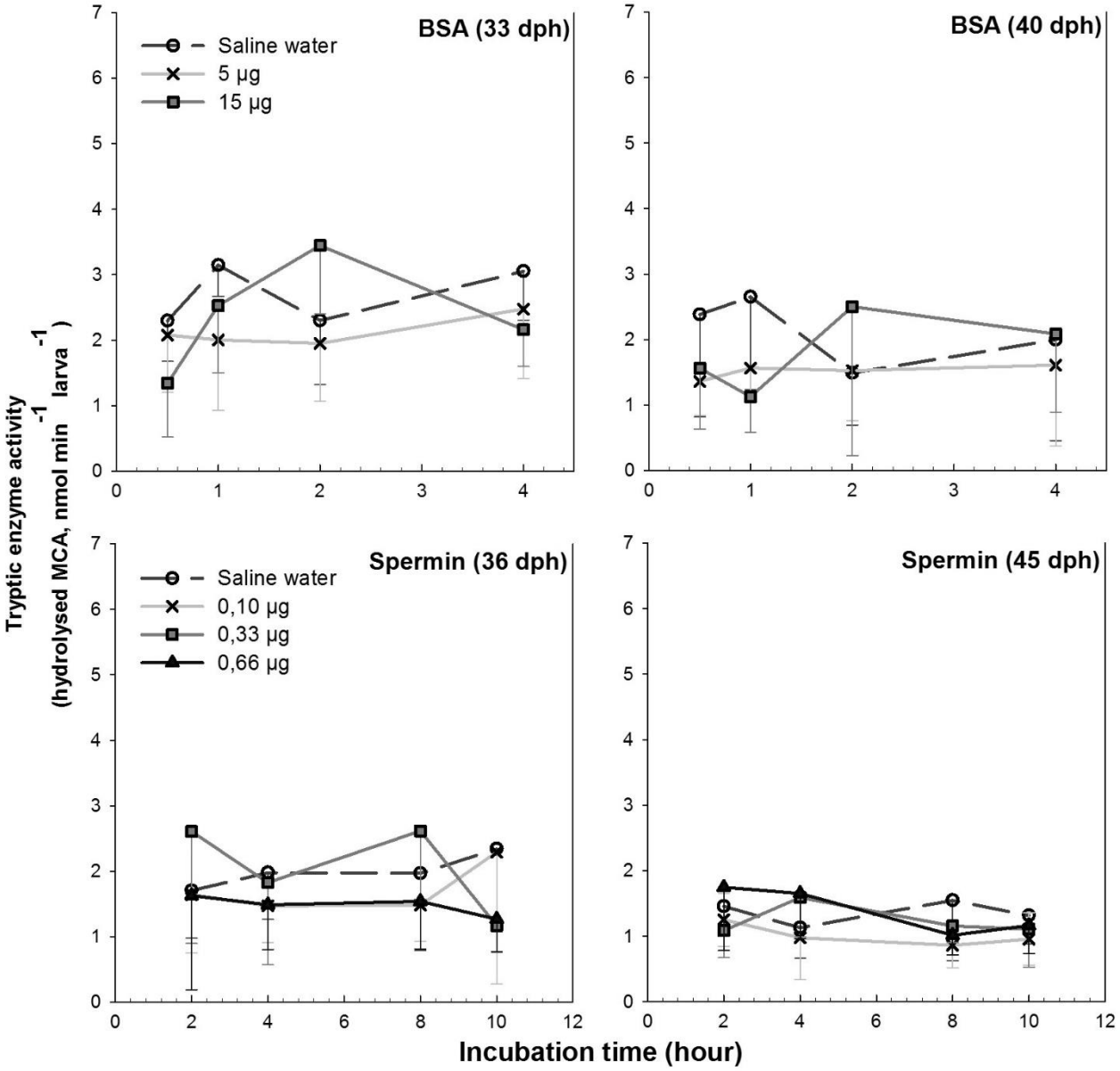

Figure II-6. Age and time response of tryptic enzyme activity (nmolMCAmin^{-1} larva^{-1}) levels of tube-fed sea bass larvae under different concentration of BSA and spermine and control at 33, 44 and 36, 45 dph, respectively. Values are given as mean (n=6/incubation time) ± SD.

Table II-2. Summary of statistically significant differences in between six different tube-fed substances, concentration, incubation time and age in sea bass larvae (mult comp test, $p < 0.05$).

Statistical Test	Substance	Concentr.	Inc. time (h)	Age (dph)	Differences of mean tryptic activity	p-value
T1	Calanus Meal	3 µg	4	60	2.2735	<0.001
	PHA	0.001%	4	59	2.8295	<0.001
	PHA	0.003%	4	59	0.1534	<0.001
T2	MiniPro	1 µg > Control	4	60	1.6526	<0.001
T3	Calanus Meal	3 µg	4 > 0.5	60	1.6334	0.0172
	PHA	0.006%	4 > 2	59	0.7228	0.0129
T4	Calanus Meal	3 µg	4	60 > 44	3.2021	<0.001
	MiniPro	1 µg	4	60 > 39	3.290	<0.001
	PHA	0.001%	4	59 > 44	4.133	<0.001

4. Discussion

The present study investigated the potential of various substances to increase tryptic enzyme activity and thus, bridge the gap in digestion deficiencies in the early larval stages. Six trigger substances were tested via tube-feeding method in sea bass larvae in various age groups which are supposed to show a clear response in tryptic enzyme activity and the results can be applied to first feeding stages.

Tryptic enzyme activity increased after tube-fed of the plant protein phytohemagglutinin (PHA), Calanus meal, protein concentrate and the extract of MiniPro in sea bass larvae. It was also demonstrated that the injected substances, their concentration and incubation time on different age have a significant impact on the amount of tryptic enzyme activity in the gut of sea bass larvae.

The general trends showed that the tryptic activity of control groups (1.99±0.29) yield similar activities as injected substance in younger larvae groups (2.07±0.28). Similar patterns and amounts of tryptic enzyme activity were obtained with saline water injected Atlantic cod larvae too (Tillner et al., 2013b). A possible explanation would be the mechanical stimulation via stretch receptors in gut by the volume of the injected substance (Pedersen and Andersen, 1992). This was verified as the untreated larvae groups which were sampled as an additional control at the same times as treated individuals showed lower tryptic activity than larvae from the control group (data not shown). Throughout the experiment, tryptic enzyme activity of the control group was lower in older larvae compared to larvae which were injected with potential trigger substances. This might be due to an increased digestive capacity with larval age as a result of increased enzymatic activity associated with the more developed digestive tracts of older larvae (Kolkovski et al., 1993).

The selected doses of PHA administrated in this study (0.001%, 0.003% and 0.006% of fresh wet weight) were higher than previously employed in Atlantic cod larvae (0.0001% of fresh wet weight) (Tillner et al., 2013b). However, it is in the same range (0.003%) as administered to halibut and herring larvae for assessing the interactions between cholecystokinin and tryptic enzyme activity in the gut (Drossou, 2006). Positive responses with 0.003% concentration were obtained in a study on larvae of Atlantic salmon (*Salmo salar L.*) by Pendás et al. (1993). This can be due to the differences between species with a straight (herring), a rotated (halibut) and a coiled (cod) gut with regard to the reaction time of the tryptic enzyme activity in response to an injection (Pedersen, 1984; Rojas-García et al., 2011; Tillner et al., 2013a). In the present study, although at 31 dph the 0.001% and 0.003% concentrations and, at 44 dph the 0.003% concentration showed slightly increase in tryptic enzyme activity, there were no significant differences with respect to the control groups. Only at 59 dph, the 0.001% concentration of PHA showed a significant increase in tryptic enzyme activity in sea bass larvae. In follow-up tube-feeding experiment in sea bream (Sparus aurata) larvae at 39 dph showed positive increase with 0.003% concentration, other than age group 22 dph or concentrations (0.005, 0.001 and 0.009% of fresh wet weight (Zeytin et. al, in preparation). In theory, it can be expected that if there is no significant increase in tryptic enzyme activity over the incubation time, those time points should be collapsed across different concentrations. Nevertheless, considering previous studies and the data from the present experiments, it was confirmed that the effect of PHA is age and concentration dependent. One possible reason for negative effects could be because of the anti-

nutritional effects of plant proteins (Kluess et al., 2007; Linderoth et al., 2006), the high inclusion levels that can cause imbalances in the amino acid profiles (Kaushik and Seiliez, 2010). Another reason could be the plant derived substances as trigger of digestive enzyme in early stages of marine larvae can be tough to break down and digest since larvae have a shorter intestine. Therefore, to achieve positive and continues increases of tryptic enzyme activity, the marked dose-response effect of PHA on tryptic enzyme activity during ontogenetic development of sea bass larvae should be considered when formulating microdiets for species specific requirements.

Studies about the addition of protein concentrate in weaning diets suggests that the dietary protein level and chain length plays a big role on digestion by adequate age-specific nutritional requirements (Cahu et al., 2004; Cahu et al., 1998; Carvalho et al., 1997; Hamre et al., 2007; Kolkovski, 2001; Zambonino Infante et al., 2008). The protein concentrate that has been used in this study is a mix of marine peptide combinations. The high protein content of the solution obviously has a stimulating effect on trypsin secretion which varies slightly depending on the age and concentration. The lowest concentration (5 µg) tested seems to have the most distinct effect on trypsin secretion. Certainly, the different protein sources, the plant protein PHA and the animal protein product protein concentrate, seem to trigger tryptic enzyme activity for the sea bass larvae, although the ability of processing and utilising the trigger substances in different ages can be related to digestive capacity or/and to the intestinal evacuation rate of tube-fed larvae.

Increase of gut evacuation time with increase fish age and size (Wuenschel M. J. and G., 2004) might also have an effect on more trypsinogen secretion. Sea bass larvae fed with a diet containing 0.33% of spermine between 20 to 38 dph showed similar results of maturational status of the intestine compared to larvae which were fed with live prey (Péres et al., 1998). In our study, the administered dose of 0.33µg spermine showed a slightly, but not statistically significant increase in tryptic enzyme activity at 36 and 45 dph compared to the control group. It might be possible that the positive effect of spermine cannot be seen in 10 hours of incubation time and need to be validated in a long-term trial. Tryptic enzyme activity was relatively similar in larvae that tube-fed with BSA in comparison to tube-fed spermine larvae. In another study, the effects of adding bovine trypsin in diets for common carp fry and post-larval kuruma shrimp *Marsupenaeus japonicus* resulted in increased total proteolytic activity (Dabrowski et al., 1978; González-Zamorano et al., 2013). No significant increase in tryptic enzyme activity was found in

our short-term experiment.

The findings in this study support the hypothesis that selected substances can enhance the release of trypsinogen in the gut beyond the rate of control groups at specific developmental stages. A conclusion about the most favourable substances may be difficult with the results from this study, but we can estimate the different feeding schedules for marine fish larvae. Sea bass larvae from 30 to 60 dph were used as a model in this study to test potential substances. Particularly, ages up to 25 dph appear to be the critical for larval survival (Cahu et al., 2004). Knowing what to feed during "critical" period in which marine fish larvae show deficiencies in tryptic enzyme capacity will help to improve digestive capacity and eventually the survival rate. This approach creates a great potential for developing a feeding strategy for marine fish larvae according to the development of their digestive physiology which might also help to increase survival rate. Feeding them in the critical stages with the specific triggering substances might help to enhance the trypsinogen production in early stages. In agreement with the results from this study, a long-term trial with the application of substances via microdiets would shed a light and might support our findings. There may be also more visible effects and differences when the potential substance deliver continuously from the microdiet. Conclusions from this study on appropriate trigger substances and concentrations, at least for sea bass larvae, can be drawn as following: phytohaemagglutinin at 0.003% of the wet weight between 30-40 dph, protein concentrate (5-15µg) and Calanus meal (1-3µg) before and after 30 dph.

In summary, the study provides some new aspects about the potential of substances which can enhance proteolytic digestive capacity. Moreover, it was demonstrated the effects of such substances depends significantly on their concentration and age in the species which was tested in this study. The results are useful for the development of formulating new generations of microdiets in marine larvae and may open new avenues for feed formulation in marine fish larvae. Ingestion rate, leaching, *in vitro* digestibility analyses and the manufacturing of the microdiet (temperature, coating, encapsulation etc.) must be considered for the calculation of the amount of substances while formulating microdiets.

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CHAPTER III

Evaluation of Calanus meal and digestive enzymes inclusion in microdiets for sea bass larvae (*Dicentrarchus labrax*) to enhance tryptic enzyme activity

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Abstract

The potential of a copepod product (Calanus meal) as a major ingredient and digestive enzyme supplements (trypsin and pancreatin) in microdiets were tested to trigger digestive capacity in sea bass larvae (*Dicentrarchus labrax*). Tryptic enzyme activity measurement in sea bass larvae was used as an indicator for evaluating the nutritive quality and digestibility of a microdiet and the nutritional condition of sea bass larvae. Within the feeding experiment ontogenetic development of tryptic enzyme activity either fed live feed (LF) (*Artemia nauplii*, control 1), a commercial microdiet (MiniPro, Norway) (MDF, control 2), microdiet with up to 50% of fish meal replaced by Calanus meal (MDC) or in addition to the replacement, enriched with enzyme mixtures such as pancreatin (MDC+P) and with the proteolytic enzyme trypsin (MDC+T) was investigated. Additionally, the effect of supplemented enzymes, pancreatin and trypsin were tested with fish meal based microdiets in MDF+P and MDF+T, respectively. At the end of the trial period (45 days) larvae fed diets which contained Calanus meal (MDC, MDC+T, MDC+P) had significantly higher weight gain than those fed fish meal based diets enriched with proteolytic enzymes (MDF+T and MDF+P) or the control (MDF). At the end of the trial the highest survival rates (in %) of 26 % were found in the LF and MDC+P groups and followed by MDF+P, MDC+T and MDF+T. Furthermore, neither 2% of pancreatin (MDF+P) nor 1.3% trypsin (MDF+T) supplementation alone led to higher tryptic enzyme activity in sea bass larvae compared to control groups (MDF and LF group). However, the combination of pancreatin or trypsin supplementation with Calanus meal MDC+P and MDC+T showed higher tryptic enzyme activity, respectively, than without Calanus meal inclusion (MDF+P and MDF+T). These results suggest that Calanus meal and pancreatin as a mixture of various enzymes and trypsin could effectively replace fish meal in sea bass larvae diets to achieve higher growth, tryptic enzyme activity, and survival rates.

Key words: Microdiet**;** Calanus meal; *Calanus finmarchicus* Sea bass larvae; *Dicentrarchus labrax*; tryptic enzyme activity; enzyme supplementation; trypsin; pancreatin

1. Introduction

The current use of live feed (rotifers and *Artemia*) for larviculture is a labor-intensive, time-consuming process and presents substantial costs (Faulk and Holt, 2009). Thus, replacing live feed to a great extend in the culture of fry of commercially important fish species will be a great deal for the aquaculture industry. However, replacement of live feed with microdiets from the onset of exogenous feeding is still a challenge in marine fish larvae husbandry. The composition, formulation, palatability, sinking rate, size, leaching rate, colour and smell (Bengtson, 1993; People Le Ruyet et al., 1993) contribute to the microdiets acceptance and influence ingestion and digestion rates which can significantly affect larvae growth and survival (Saleh et al., 2013). In addition, the nutritional requirements of fish larvae are different from the requirements of juveniles or adults (Yúfera, 2011). This is mainly due to the organization and the developing functionality of the forming digestive system (Wallace et al 2005). Marine fish larvae digestive tracts are not fully developed at the onset of exogenous feeding. Nevertheless, their gut has a digestive capacity (Rønnestad et al., 2007) that they can digest live feed or microdiets depending availability. Kolkovski et al. (1993) suggested that live feed is ingested at a higher rate than microdiets in terms of feeding rate. In addition, live feed, with a thin exoskeleton and high water content, may be more palatable to the larvae once taken into mouth, compared with the hard, dry microdiets (Støttrup and McEvoy, 2003). Although a significant contribution of digestive enzymes to overall larval digestion capacity derived by live feed has not been demonstrated (Cahu and Zambonino Infante, 1995), it is hypothesized that the stimulation of feeding behaviour and attractiveness of live feed assists more efficient digestive process in live feed than of formulated microdiets (Conceição et al., 2010b). Another possible explanation of the limited digestibility in fish larvae could be that the pancreatic enzyme secretion is not sufficiently stimulated to utilize some of the ingredients in microdiets (Yúfera et al., 2000). Hence, providing suitable feed adapted to the digestive potential at this early larval stage is crucial. Among the pancreatic enzymes, trypsin is the most significant protease in the early larval stages and is considered to be a key enzyme in the digestive process of proteins (Ueberschär, 1995). Therefore, several studies focussed on the benefits of using proteolytic enzymes as supplements in aquaculture feeds for enhancing digestive capacity and increasing growth rate (Buchanan et al., 1997; Dabrowski and Glogowski, 1977; Divakaran and Velasco, 1999; González-Zamorano et al., 2013). These studies obtained better biological and physiological performance compared

with diets containing no enzymes, but an enzyme supplemented diet was still less effective than the live feed.

In general, digestive enzyme activities of fish are associated with innate feeding habit and diet composition (Ray, 1988). Thus, a composition should be chosen which represents practically a copy of the natural feed of fish larvae. Fish meal is still the major protein component in microdiets, as it is essential to meet the high protein requirements with an excellent amino acid profile required at this early life stage (Kaushik and Seiliez, 2010). However, there is still a bottleneck in research aimed at tackling weak growth performance and survival rate of marine larvae using microdiets (Hamre et al., 2013). Major limitations on the application of microdiets are primarily low ingestion rates and low digestibilities, as well as inadequate nutrient supply (Rønnestad et al., 1999). Marine zooplankton, as a natural feed item for many fish species, particularly at larval stage, represent viable biomass and show high potential as a source of protein and lipid (Bergvik et al., 2012) but also as a 'feed attractant' (known as group of chemicals act to stimulate feeding response, (Kolkovski et al., 1997b)). Such substances are also existing in live nauplii (Garatuntjeldsto et al., 1989; van der Meeren et al., 2008). One of the most abundant zooplankton in the North Sea, *Calanus finmarchicus* is an important nutrient source for marine fish larvae (Tande et al., 2016). Calanus and copepods have high levels of EPA (20: 5n-3), DHA (22: 6n-3) (Conceição et al., 2010b; Tande et al., 2016), vitamins and minerals. Accordingly, Calanus products (freeze-dried marine copepods, *Calanus finmarchicus*), could act as an alternative ingredient for the aquaculture industry (Colombo-Hixson et al., 2012). In addition, Calanus products could mimic the palatability of live feed.

To our best knowledge, no studies have been reported on the use of Calanus meal as fish meal substitute and the combination of digestive enzyme (trypsin and pancreatin) supplementation for enhancing digestive capacity in sea bass larvae microdiets. Therefore, the primary objectives of this study were to evaluate the impact of different microdiets and enzyme supplements on growth, survival and tryptic enzyme activity of sea bass larvae (*Dicentrarchus labrax*) throughout the first 45 days post hatch. The experimental microdiets were compared against the traditional live feed (*Artemia nauplii*) to evaluate the potential of decreasing or replacing live feeds in early stages in sea bass larvae nutrition.

2. Materials and methods

2.1. Larval rearing

Sea bass larvae (1-day post hatch, dph) were obtained from a commercial hatchery in Gravelines, France[5] and transported to the experimental facility of the GMA[6], Germany. The larvae were equally distributed to 21 green conical tanks (65 L water volume each) with a stocking density of 110 larvae L^{-1} and kept in darkness until yolk sac absorption and mouth opening at 7 dph (French technique). Larval rearing was carried out in a semi-closed recirculating system as described in Zeytin et al. (2016). Light intensity was kept at 270 lux at the water surface via illumination above the tanks and a light/dark cycle of 16:8-h was adopted. Oxygen was kept between 6.7 and 7.9 mg L^{-1} (80%-97% saturation level) throughout the experiment and the water temperature was gradually increased (0.5°C/week) from 16.5 °C to 19 °C during the experimental period. pH was measured every day (7.87±0.58). Salinity was slowly decreased from 33 to 24 psu (1 psu/4 days) and kept constant until 45 dph. The seawater was filtered (polyester filter cartridges 25µm) and treated with UV light (55 Watt UVC). A surface skimmer was installed in each small tank to constantly remove oil-like films from the water surface in order to facilitate swim bladder inflation. A protein skimmer was installed to remove organic debris from the basal tank. Additionally, the water surface in the rearing tanks was skimmed with a paper towel and accumulations of feed on the bottom of the tanks were siphoned frequently. All rearing tanks were equipped with a water inlet below the water surface and aerated with an air tube with gentle bubbles from the centre of the bottom. Green water technique was applied, and each tank received 1 mL microalgae *Nannochloropsis sp*. paste (600000 cells/ml, BlueBioTech, Germany) pre-mixed with seawater two times a day at 07:00 and 20:00, respectively.

2.2. Experimental diets (microdiets)

Five experimental microdiets (MD) and one commercial microdiet (MDF) as well as one live feed (LF, feeding only with *Artemia nauplii* up to the end of the experiment) were used as control groups. The production of these microdiets (MD) was done by the Norwegian manufacturer MiniPro A/S. Diet formulations of the experimental microdiets are shown in Table

[5] Ecloserie Marine de Gravelines, 59820, France
[6] Gesellschaft für Marine Aquakultur mbH, Hafentörn 3, 25761, Büsum/Germany

1. In comparison to the MDF, 50% of the standard hydrolysed fish meal was replaced by a meal of freeze-dried marine copepods (*Calanus finmarchicus*) in the experimental diet MDC, 49.2% in MDC+T and 48.7% in MDC+P. In addition to the replacement, MDC+P was enriched with enzyme mixtures (pancreatin) and MDC+T with the proteolytic enzyme trypsin. In order to evaluate the influence of different protein sources (fish meal and Calanus meal) in combination with enzyme supplementation efficiency, pancreatin and trypsin were tested in fish meal based microdiets MDF+P and MDF+T diets, respectively. Amino acids such as Glycine, Betaine and Arginine (in the ratio of 1:1:1) were added in all experimental microdiets as attractants according to a previous study on the effect of free amino acids on tryptic enzyme activity of seabass larvae (in prep.). All experimental groups were carried out in triplicates.

2.2.1.Calculation of amount of trypsin and pancreatin in experimental microdiets

The amount of trypsin and pancreatin in diets were calculated according to previous tube-feeding and *in vitro* experiments in sea bass larvae (Zeytin et. al. 2018, under review). In order to prove that the enzymes added were not destroyed during feed processing; few test exemplary microdiets production were realised by MiniPro A/S, Norway as a sample to verify the amount of trypsin to be supplemented in diets previous to the final production of experimental microdiets. The test MD samples were prepared following the commercial fish meal based MiniPro product (MDF). Trypsin was supplemented to the MDF in a concentration of 10 g per 1.5 kg dry matter. During the production process this amount of trypsin was diluted in 1 Liter of liquid phase.

Table III-1. Ingredients (g kg^{-1} dry matter) of the experimental microdiets for sea bass larvae. F indicates fish meal, C: Calanus meal, T: Trypsin and P: Pancreatin. MDF: commercial MiniPro product based on fish meal, MDF+T: Fish meal+Trypsin, MDF+P: Fish meal+Pancreatin, MDC: microdiet with Calanus meal (C), MDC+T: Calanus meal+Trypsin, MDC+P: Calanus meal+Pancreatin.

	Experimental diets					
	MDF	**MDF+T**	**MDF+P**	**MDC**	**MDC+T**	**MDC+P**
Ingredients (g kg^{-1} dry matter)						
Fish protein hydrolysat[1]	794	756	749	384.5	378	374.5
Calanus meal[2]	0	0	0	384.5	378	374.5
Trypsin[3]	0	13	0	0	13	0
Pancreatin[4]	0	0	20	0	0	20
Attractant[5]	0	25	25	25	25	25
Carbohydrate[6]	62	62	62	62	62	62
Lecitin[7]	60	60	60	60	60	60
Algamac[8]	53	53	53	53	53	53
Rape seed oil	20	20	20	20	20	20
Carophyll pink[9]	7	7	7	7	7	7
Vitamin and mineral mix[10]	4	4	4	4	4	4

[1] Seagarden AS Husøyvegen 278, 4262 Avaldsnes
[2] Calanus® Freeze Dried, Calanus AS, www.calanus.no
[3] Sigma-Aldrich, 85450C- porcine trypsin 1:250
[4] Sigma-Aldrich; Pancreatin from porcine pancreas, P1750 Batch: SLBF6528V
[5]Arginine, Glycine: Carl Roth, Product no: 3144.1, 3187.1 and Betaine: Sigma-Aldrich, Product no: B2629
[6] Selvig, Bekkajordet 9 3189 Horten
[7] Brenntag Nordic Po Box 144 Torvila 2 1740 Borgenhaugen
[8] Algamac 2000, Product from USA, www.aquafauna.com/Diets-AlgaMac-2000.htm
[9] Nature identical astaxanthin, Roche, Product code: 04 1212 0
[10] Cambridge Commodities 78 Lancaster Way Business Park Ely CB63NW United Kingdom
[*] Nitrogen free extract=1000- (crude protein + crude lipid + crude ash)

2.3.Feeding regimes

From the start of exogenous feeding (7 dph) all groups were fed with *Artemia nauplii* (Ocean Nutrition, Micro Artemia Cysts, +/- 430 µm) three times a day (at 8 a.m., 14 and 20 p.m.) with a density of 5 *Artemia* ml^{-1}/feeding event and increased gradually to 10 *Artemia* ml^{-1}/feeding event until 15 dph. At 15 dph three tanks were randomly assigned to each different experimental diet (MDF, MDF+T, MDF+P, MDC, MDC+T, MDC+P and live feed (LF) (*Artemia nauplii)* as shown in Fig.III- 1. Feeding of different microdiets was conducted by means of an automatic microdiet dispenser system (AMD-system®). The AMD-system allowed administering microdiets periodically in adjusted quantities by the number of shots per feeding event. Feeding frequencies and amounts of microdiets with number of shots were adjusted according to larval age and aimed at MDs present in the water column most of the time during the feeding period. Period of feeding events during the experiment were from 8 a.m to 20 p.m. Each diet dispersed well on the surface of the water and sank gradually. During 10 days of co-feeding period (between 15-25 dph), *Artemia* density in microdiet treatments was gradually reduced and, from 25 dph onwards, larvae were fed on microdiets exclusively. The amount of feed administered to each tank was determined every day by weighing the initial and remaining amounts of MDs in the trays of the AMD after 24 h. The total amount of MDs administered from co-feeding to the end of the experiment can be seen in Table.2. The experimental diet size used were <150 µm for larvae from 15 to 30 dph, and gradually changed to size 200–300 µm for larvae from 35 dph onwards. Live feed groups were fed with gradually increased amount of *Artemia* and enriched *Artemia* (S-Presso, INVE) from 25 dph until 45 dph (see Fig.III-1). The experiment was terminated after 45 days post hatch (or abbreviate as dph).

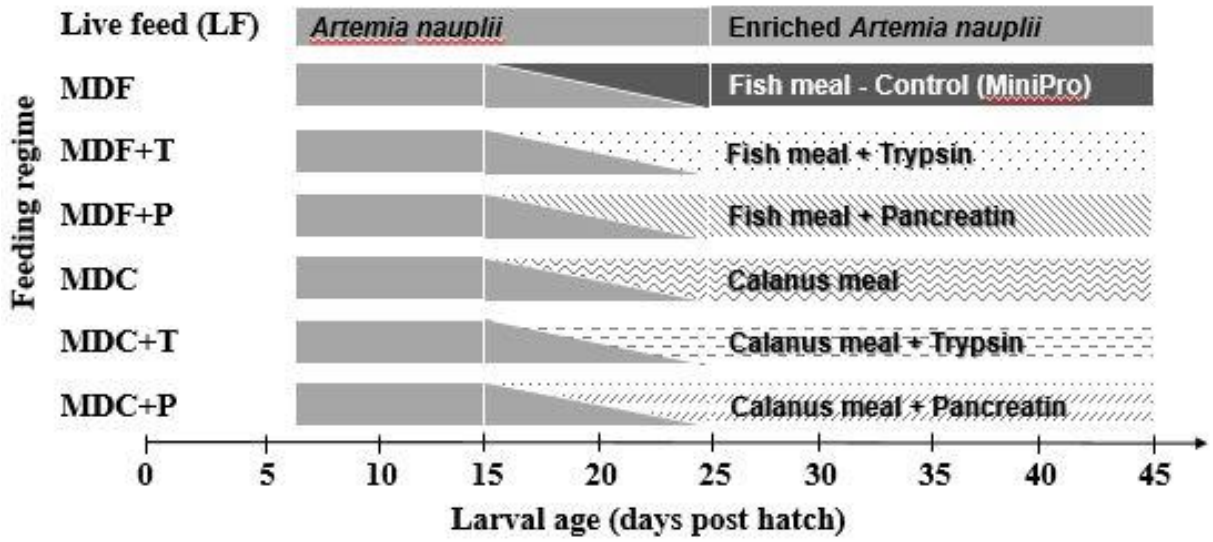

Figure III-1. Feeding regime of different groups of sea bass larvae over the experimental period of 45 days. Two different feeding regimes defined as control: standard commercial microdiet MiniPro (MDF) as Control 1 and a continuous feeding with live feed *Artemia* nauplii as Control 2. Fading areas between 15 to 25 dph indicate the co-feeding period from the live feed to the experimental microdiets. Groups fed with microdiets were fed by an automatic feeder, whereas the live feed groups fed by an administration of *Artemia nauplii* three times a day.

Table III-2. Feeding frequencies and amounts of microdiets in different age groups of sea bass larvae. The total amount of feed referred to an average value for all test tanks/day/24 h.

Larval age (dph)	Amount of feeding events (and shots per event)	Feeding frequency (min)	Total amount of feed /tank/ day (g)
15-20	15 (1)	54	0.45
21-25	30 (1)	28	0.9
26-30	60 (1)	15	1.8
31-40	60 (2)	15	3.6
41-45	60 (3)	15	5.4

2.4.Analyses of the macro nutrients

The actual macro nutrient composition of the microdiet was analysed following standard procedures of the EU guideline (EC) 152/2009 (European Union, 2009a). Dry matter of samples was determined after drying at 103 °C until constant weight remained stable. Ash content was analysed after 4 h incineration at 550 °C in a combustion oven (P300; Nabertherm, Lilienthal, Germany). Crude protein content (N×6.25) was determined by the Kjeldahl method (InKjel 1225M, WD 30; Behr, Düsseldorf, Germany), crude lipid content was analysed by means of hydrolysis with hydrochloric acid followed by a petroleum ether extraction with a Soxleth extraction system (Soxtherm, Hydrotherm, Gerhardt, Königswinter, Germany). Furthermore, gross energy was measured in a bomb calorimeter (C 200; IKA, Staufen, Germany). Dietary nutrient composition of the experimental microdiets are shown in Table 3.

Table III-3. Macro nutrient composition (g kg^{-1} dry matter) and dietary tryptic enzyme activity (hydrolised MCA, nmol min^{-1} mg) of the experimental microdiets tested in feeding trial for sea bass larvae.

LF: Live feed (*Artemia nauplii*), F indicates fish meal, C: Calanus meal, T: Trypsin and P: Pancreatin. MDF: commercial MiniPro product based on fish meal, MDF+T: Fish meal+Trypsin, MDF+P: Fish meal+Pancreatin, MDC: microdiet with Calanus meal (C), MDC+T: Calanus meal+Trypsin, MDC+P: Calanus meal+Pancreatin. Data in macro nutrient composition are presented as mean (n=2) ± SD. Values with different superscript letters are significantly different ($p < 0.05$). Data in dietary tryptic enzyme activity are presented (n=3) ± SD. Values with different superscript letters are significantly different ($p < 0.05$).

	Experimental microdiets						
	LF	**MDF**	**MDF+T**	**MDF+P**	**MDC**	**MDC+T**	**MDC+P**
Nutrient composition (*g kg^{-1} dry matter*)							
Dry matter	957.5±0.25^{a}	929.2±0.02^{b}	956.1±0.04^{a}	931.4±0.05^{b}	891.5±0.08^{d}	929.8±0.04^{b}	902.9±0.05^{c}
Crude protein	531.2±0.21^{e}	647.2±0.01^{a}	606.1±0.35^{c}	574.2±0.31^{d}	572.7±0.30^{d}	623.3±0.38^{b}	580.1±0.43^{d}
Crude lipid	180.1±0.04^{bc}	118.2±0.13^{e}	145.5±0.31^{d}	188.1±0.45^{ab}	193.6±0.03^{a}	114.8±0.20^{e}	175.9±0.31^{c}
Crude Ash	150.3±0.24^{b}	129.8±0.05^{d}	139.4±0.02^{c}	132.3±0.00^{d}	110.6±0.00^{e}	167.3±0.02^{a}	128.9±0.05^{d}
NfE*	138.4	104.8	109	105.4	123.1	94.6	115.1
Gross energy (MJ kg^{-1})	21.94±0.23^{c}	22.0±0.02^{c}	22.7±0.13^{b}	20.7±0.02^{d}	22.8±0.03^{b}	21.9±0.03^{c}	23.7±0.13^{a}
Dietary tryptic enzyme activity (hydrolised MCA, nmol min−1 mg)	0.1±0.02^{a}	88.0±4.60^{c}	78.06±2.45^{d}	1.5±0.34^{a}	11.6±0.32^{b}	11.6±0.29^{b}	

2.5. Sampling, sample preparation and analyses of tryptic enzyme activity

Samples of five individual sea bass larvae for analyses of tryptic enzyme activity, total length and fresh wet weight determination were taken every five days from each replicate tank. Additional five individual larvae from each tank were sampled at 18 dph to evaluate the effects on the indicators in the transition period from live feed to microdiets on the same parameters as above. All samples were collected before the first feeding at a sampling day to ensure that larvae were not fed, and their gut was empty. This was also confirmed during the measurement under the microscope. Samples for tryptic enzyme activity were immediately preserved at -80°C. The total length of each larva (mm, from the tip of snout to the posterior margin of the body) was measured under the microscope using a millimetric scale. The wet weight of each larva was measured with a microbalance scale (Sartorius Cube, ± 1μg). At the termination of the trial (45 dph), all remaining larvae from each tank representing each dietary treatment larvae were counted, and total survival was calculated per tank as a percentage of the initial number of larvae.

The following parameters were calculated:

Fulton condition factor (FCF) = 100*final body weight*final body length^{-3}

Specific growth rate (SGR), % per day:100 × (ln FBW-ln IBW)/experimental days,

FBW: Final body weight (gr), IBW: Initial body weight (gr)

The tryptic enzyme activity of each individual larva was analysed using a fluorescence technique according to (Ueberschär, 1993). The synthetic fluorescence substrate used here was Nα-benzoyl-L-arginine-methyl-coumarinyl-7-amide-HCl (BACHEM, Art.No: 4002540.0250) and the buffer was TRIS–HCl (0.1M, pH 8) (MERCK, Art.no.1083820500) including $CaCl_2 \times 2H_2O$ (0.02M) (MERCK, Art.no. 2382). All samples were kept on ice during the preparation of individual larval samples for trypsin enzyme activity measurement in order to avoid any proteolytic activity. Each frozen larva was placed on a petri dish stored on crushed ice to thaw slowly and following thawing rinsed with distilled water. Each individual was transferred into a 1.5 ml Eppendorf vial and homogenized in 250μL ice-cold TRIS-Buffer by using a motorized pestle. The homogenate was centrifuged for 45 min (4110xg, 0°C) to settle the tissue fragments from larvae; subsequently the supernatant was used for the analysis of tryptic enzyme activity. Tryptic enzyme activity is expressed as the amount of hydrolysed substrate (μmol MCA) per

minute and larva. A MCA standard curve was used to calculate the tryptic activity in the samples.

Dietary tryptic enzyme activity of the experimental microdiets was measured as described in 2.2.1 and are presented in Table 3.

2.6.Statistical analyses

The statistical software R (2017) was used to evaluate the data. The data evaluation started with the definition of an appropriate statistical mixed model (Laird and Ware, 1982; Verbeke and Molenberghs, 2000). The data were assumed to be normally distributed and to be heteroscedastic due to the different feeding regimes and ages. These assumptions are based on a graphical residual analysis. The statistical model included feeding regimes (MDF, MDF+T, MDF+P, MDC, MDC+T MDC+P and LF) and age, as well as their interaction term as fixed factors. The tank was regarded as random factor. Based on this model, an analysis of variances (2-way ANOVA) was conducted, followed by multiple contrast tests (e.g., see Bretz et al., 2011, Schaarschmidt and Vaas (2009) to provide more information on which parameter cause the significance. Significance was set at a = 0.05 for all analyses.

3. Results

All the experimental diets were well accepted by the sea bass larvae according to the microscopic observations (gut fullness, data not shown).

3.1. Growth performance

At the end of the trial (45 dph), the maximum length of larvae was recorded in the live feed group, following MDC+T (15.24 mm) and MDC, respectively (Fig.III-2, Table.III-4). The differences between the microdiets are even more pronounced (Fig.III-3) in larval weight than in larval length with increasing age.

Larvae fed diets containing Calanus meal (MDC+P, MDC+T and MDC) had significantly higher growth in weight than those fed corresponding fish meal based diets (MDF+P and MDF+T) or the control (MDF). In general, the effects of the feeding regimes became more significant after about 35 dph (after 10 days of completely microdiets feeding). Differences in weight gain were also reflected in the specific growth rate (SGR). The SGR of the larvae fed with LF was

significantly higher than those fed diets MDF+P, MDF+T and MDF between 25 and 45 dph. The condition factor was not significantly different in all feeding groups.

The results of the two-way ANOVA indicated a significant main effect for different protein source (MDC, MDF) ($p<0.05$) and a significant main effect for different enzyme supplementation (MDF+T, MDF+P, MDC+T and MDC+P). Additionally, the results show a significant interaction between both protein source and enzyme supplementation ($p<0.05$), indicating that any difference in growth were dependent upon which protein source and which enzyme were added (Table 4).

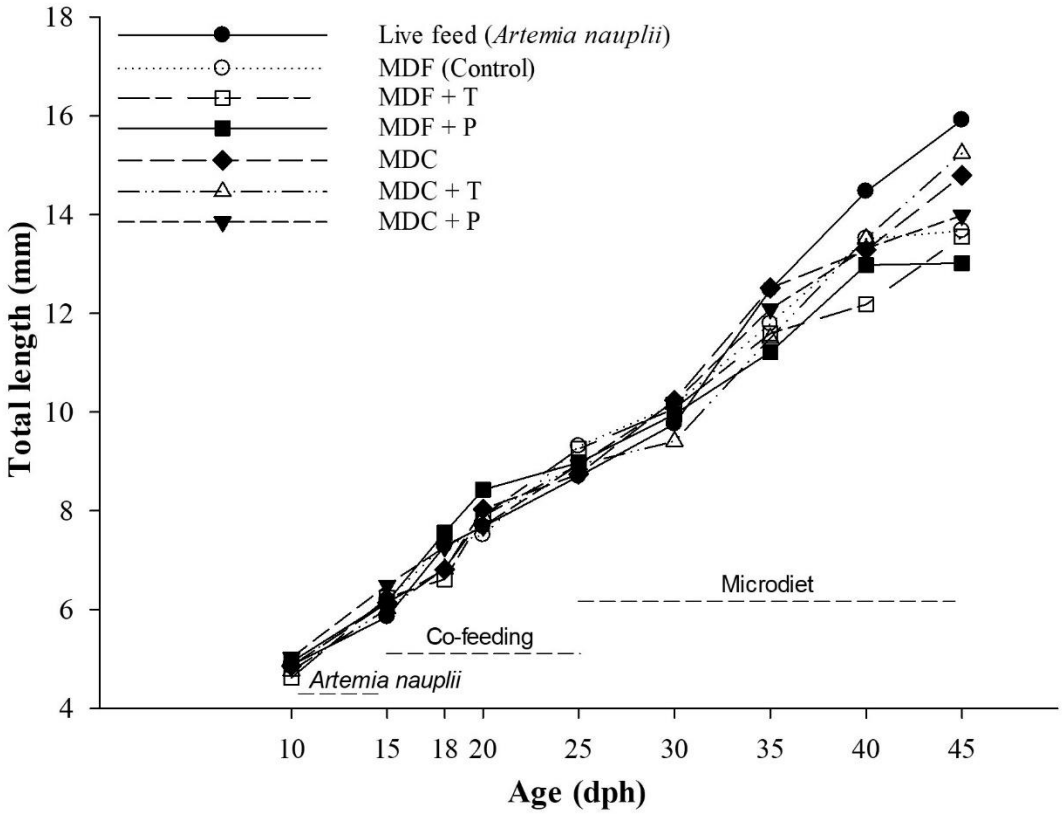

Figure III-2. Total length with increasing age between different feeding regimes in sea bass larvae. Data are presented as mean (n=5 individuals/tank, n=3 tanks/treatment). Arrow indicate the first administration of live feed (Artemia nauplii) in all groups. Dash lines indicate the feeding period: control group which fed only with Artemia nauplii from beginning to end of the experiment), co-feeding group from 15 to 25 dph and the group which fed only with microdiets from 25 to 45 dph. No significant differences in length were found between different feeding regimes during the experimental period ($p< 0.05$).

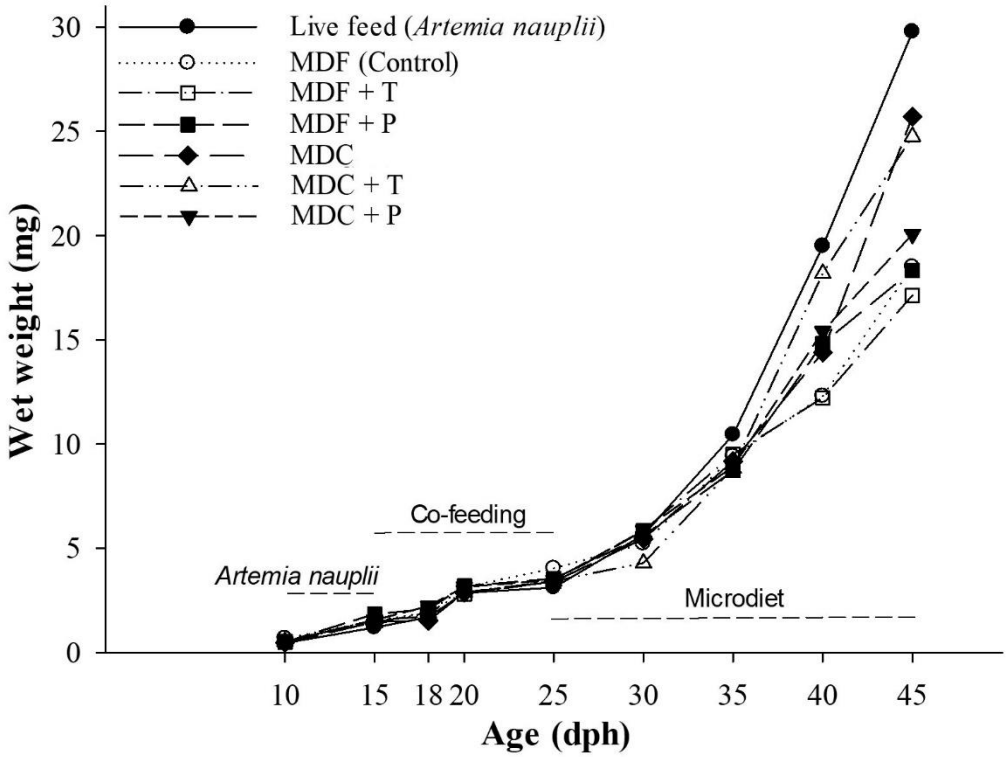

Figure III-3. Wet weight with increasing age between different feeding regimes in sea bass larvae. Data are presented as mean (n=5 individuals/tank, n=3 tanks/treatment). Arrow indicate the first administration of live feed (*Artemia nauplii*) in all groups. Dash lines indicate the feeding period: control group which fed only with *Artemia nauplii* from beginning to end of the experiment), co-feeding group from 15 to 25 dph and the group which fed only with microdiets from 25 to 45 dph.

Values with significant differences are shown in Table 4.

3.2. Survival

The highest relative survival rates were observed in the LF group and MDC+P, with comparable values of 26%, and followed by MDF+P, MDC+T and MDF+T with survival rates of 22%, 20% and 19% respectively (Table 4).

Table III-4. Growth performance of sea bass larvae fed experimental diets. IBW: Initial body weight (mg), FBW: Final body weight (mg), FL: Final length (mm), FCF: Fulton condition factor, SGR: Specific growth rate. Data are presented as mean (n=5 individuals/tank, n=3 tanks/treatment) ± SD for three replicates. Values with different superscript letters are significantly different ($p < 0.05$).

	LF	MDF	MDF+T	MDF+P	MDC	MDC+T	MDC+P
IBW at 10 dph	0.50 ± 0.12^{ab}	0.68 ± 0.12^{a}	0.53 ± 0.08^{ab}	0.50 ± 0.09^{ab}	0.47 ± 0.09^{b}	0.51 ± 0.15^{ab}	0.53 ± 0.15^{ab}
FBW at 45 dph	31.80 ± 6.63^{a}	18.50 ± 6.79^{b}	17.13 ± 10.40^{b}	18.34 ± 9.54^{b}	25.71 ± 9.25^{ab}	23.06 ± 8.04^{ab}	20.07 ± 7.59^{ab}
FL at 45 dph	15.9 ± 2.17^{a}	13.7 ± 1.31^{a}	13.6 ± 1.55^{a}	13.0 ± 1.04^{a}	14.8 ± 1.57^{a}	15.2 ± 1.98^{a}	14.0 ± 1.95^{a}
FCF[4]	0.87 ± 0.40^{a}	0.74 ± 0.30^{a}	0.73 ± 0.47^{a}	0.84 ± 0.47^{a}	0.86 ± 0.47^{a}	0.69 ± 0.35^{a}	0.80 ± 0.46^{a}
SGR between 15-25 dph	8.16 ± 4.34^{a}	9.93 ± 2.43^{a}	8.06 ± 3.96^{a}	6.67 ± 3.80^{a}	8.31 ± 2.40^{a}	9.18 ± 4.54^{a}	8.01 ± 3.32^{a}
SGR between 15-45 dph	10.42 ± 1.32^{a}	8.26 ± 1.32^{ab}	7.92 ± 1.54^{b}	7.37 ± 1.65^{b}	9.45 ± 1.43^{ab}	9.02 ± 1.46^{ab}	8.32 ± 1.69^{ab}
SGR between 25-45 dph	11.55 ± 1.82^{a}	7.42 ± 2.03^{b}	7.85 ± 2.20^{b}	7.72 ± 3.06^{b}	10.02 ± 2.49^{ab}	8.95 ± 1.78^{ab}	8.47 ± 2.88^{ab}
Survival rate at 45 dph (%)	26	19	19	22	14*	20	26

* Although a good growth rate was achieved in MDC, there were problems with the oxygen supply in one of the replicate groups in the meantime. Therefore, survival rate for MDC group was calculated only for 2 replicate group.

3.3.Tryptic enzyme activity of sea bass larvae

Tryptic enzyme activity was significantly higher in the LF group compared to other feeding groups from 25 dph onwards (Fig. III-4), whereas the other groups showed similar levels. Only MDC showed a clear peak at 35dph.

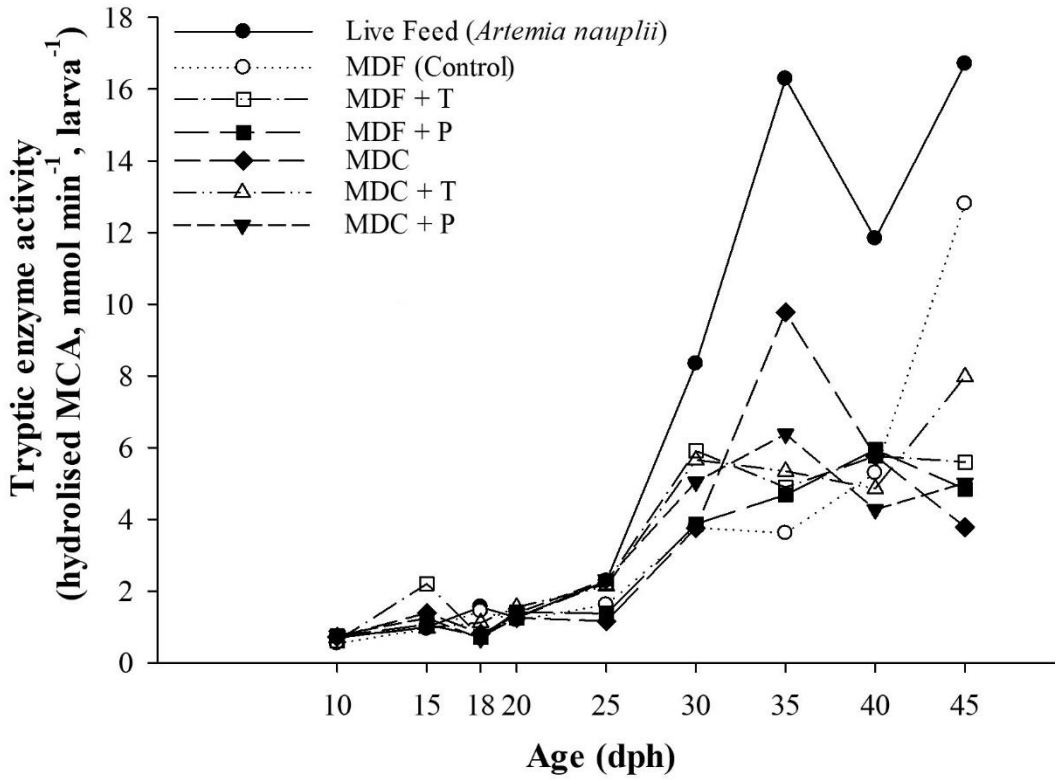

Figure III-4. Tryptic enzyme activity (hydrolised MCA, nmol min^{-1} $larva^{-1}$) with increasing age between different feeding regimes. Data are presented as mean (n=5 individuals/tank, n=3 tanks/treatment). Values with significant differences are shown in Table 4.

In general, tryptic enzyme activity increased with the increase of larval size during ontogeny. The increasing trend between larval length and tryptic enzyme activity is shown in Fig. III-5. Larvae between 4 and 9 mm length showed a similar range of tryptic enzyme activity among all groups. The tryptic enzyme activity increased in larvae between 9 and 17 mm length with regard to different feeding regimes. The trypsin activity at individual larvae (length) showed higher fluctuations (Fig. III-4).

Larvae fed with microdiets enriched with proteolytic enzymes but without Calanus meal (MDF+P and MDF+T) generally showed lower trypsin activity (Fig.III-4, 5). On the other hand, larvae fed microdiets enriched with proteolytic enzymes and Calanus meal showed higher values (MDC+P and MDC+T) in larvae between13.5 and 17.5 mm.

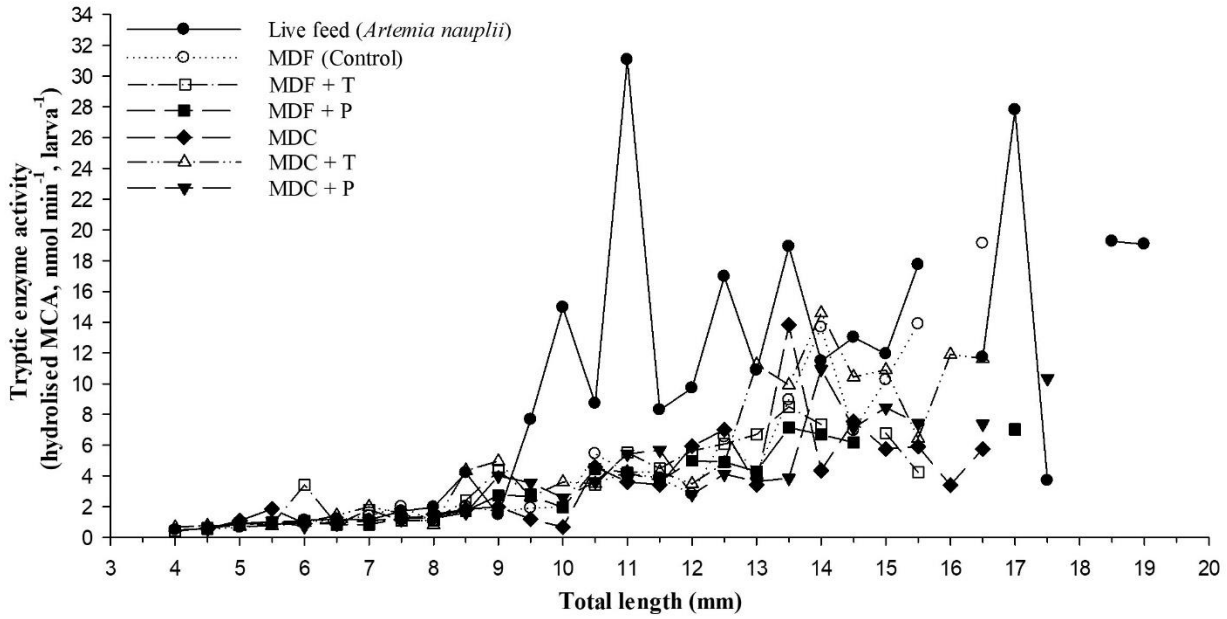

Figure III-5. Tryptic enzyme activity for length classes (total length, 0.5 mm precision, 4.0 – 4.5, 4.5 – 5.0 etc.) of larval sea bass. Data are presented as mean (1-17 larvae per length class per treatment). No significant differences were found ($p < 0.05$).

4. Discussion

This study describes the combined effects of partial substitution of fish meal by Calanus meal and the effect of enzyme supplementation in microdiets on growth performance, survival and tryptic enzyme activity in sea bass larvae. The replacement of fish meal in MDF by Calanus meal of up to 50% (MDC+P, MDC+T and MDC) as a major component in formulated microdiets did not affect the diet acceptance nor feeding behaviour in the sea bass larvae. Prior to our study, the nutritional evaluation of *C. finmarchicus* meal as a feed ingredient was tested in Atlantic halibut juveniles (Colombo-Hixson et al., 2012). Still, the present study reports the first-time data of Calanus meal as an alternative nutrient source on sea bass larval diets.

Inclusion of Calanus meal up to 38% resulted in high growth rates, particularly in larvae fed MDC+P, MDC+T and MDC than in larvae fed commercial diet MDF by the end of experiment. Similarly, Colombo-Hixson et al. (2012) found high growth performance in halibut juveniles fed a diet containing 24% *C. finmarchicus* meal inclusion. Digestibility is a key parameter in defining larval growth performance as well as survival rate while testing an alternative ingredient in marine fish larvae diets (Conceição et al., 2010a). In this study, the final survival of 26%, total

length of 15.2±1.98 mm and total weight of 25.71±9.25 mg in sea bass larvae fed diets containing Calanus meal (in MDC+P, MDC+T and MDC, respectively) was higher than fish larvae fed fish meal based MDF diets. MDC+P with 37.5% Calanus meal and 2% of pancreatin supplementation achieved the same survival rate as the control with LF group (*Artemia Nauplii*) at 45dph. Survival rate of 26% reported in this study was higher than previously reported (18%) for sea bass larvae co-fed with commercial diets from 15 dph on as in our study (Suzer et al., 2007). However, this same study reported higher weight and length values (34 mg, 18 mm). In contrast to growth performance, tryptic enzyme activity in fish larvae was lower in cited group (group with 18% survival and, higher length and weight values) than in MDC+P and MDC+T. This could be attributed to the addition of enzymes in our diets. However, Suzer et. al., 2007 did not assess the effect of the added enzymes on microdiet assimilation and absorption. In contrary, Kolkovski (2001); Kolkovski et al. (1993) have demonstrated that the addition of dietary exogenous enzymes improved microdiet assimilation and absorption in gilthead sea bream larvae. Based on our results, we propose that the insufficient digestive enzyme activity in the early stage of sea bass larvae were enhanced most likely by the combination of stimuli effect of supplemented digestive enzymes and the experimental microdiet ingredients. Nevertheless, there is a need to elaborate further and in detail the pathway of digestion of experimental microdiets and tryptic enzyme secretion.

Generally, the highest mortality occurs between 15-25 dph when marine fish larvae show deficiencies in proteolytic pancreas enzymes, especially in tryptic enzyme activity (Galaviz et al., 2011; Rønnestad et al., 2013; Ueberschär, 1995; Zambonino Infante and Cahu, 2001). In this critical phase the supplementation of pancreatin or trypsin may help to bridge the lack of digestive capacity. However, in the present study, neither 2% of pancreatin (MDF+P) nor 1.3% trypsin (MDF+T) supplementation without Calanus meal led to better results in terms of tryptic enzyme activity compared to control groups (MDF and LF group) but higher survival in MDC groups. This could be caused by the high trypsin leaching rate of MDF+T and MDF+P compared to Calanus meal based microdiets (data not shown). It can be also argued, that the individual concentration of either pancreatin or trypsin in the microdiets was too low to yield an effect, but the combination of pancreatin and trypsin supplementation with Calanus meal MDC+P and MDC+T showed better results than without Calanus meal recipes (MDF+P and MDF+T), respectively. The relatively better performance of the MDs with a combination of Calanus meal and supplemented digestive enzymes could be due to the favourable fatty acid profile of Calanus

meal and trigger effects of the pancreatin and trypsin supplementation in the early stage of sea bass larvae during the critical phase. The added Calanus meal is based on freeze-dried *Calanus finmarchicus* (Calanus® Freeze Dried). Of the total fatty acid content, 22% were omega-3 fatty acids, including 7% of EPA (20: 5n-3) and 5.4% of DHA (22: 6n-3) (Calanus® specification sheet). There was a concern regarding the significant amount of wax esters in the product, since they may not be effectively utilized, particularly in juvenile fish (Olsen et al., 1991). Nevertheless, studies have shown that many species of fish have the capacity to digest wax esters without any detrimental change in growth or body lipid composition (Benson et al., 1975; Mankura et al., 1984; Olsen et al., 2004).

The Algamac-2000 (spray-dried cells of marine algae *Schizochytrium* sp.), which is present in all MD recipes, should provide the fish larvae with the necessary fatty acids in the right proportions and quantities. However, even after the addition of the Algamac-2000, DHA could still be a limiting factor. However, future research need to consider fatty acid demand of ongrowing fish larvae. The Algamac-2000 has a DHA content of only 27%. It is possible that the Calanus product provided further DHA, which leads to the positive effects in the growth of the sea bass larvae. In the standard formulation of MiniPro, the algae concentrate Algamac-2000 contains only 1.4 g DHA/100 g dry matter in the feed. Gisbert et al. (2005) obtained the best results in terms of growth, survival and maturation of digestive system in group fed with 2.3% of EPA and DHA in diets for sea bass larvae. An increase in the DHA concentration to 2.0 g DHA/100g dry matter in the feed might be appropriate to further increase survival rates. Lipid-rich feed was particularly advantageous from day 35 onwards. To improve the MDs, the lipids in the feed should be increased to over 20%. In the literature even up to 26% lipid content in the fish larvae diet is proven to result in improved survival rates and improved growth (Cahu et al., 2003).

Tryptic enzyme activity is an appropriate indicator for digestibility of feed in the early stages of marine fish larvae (Lauff and Hofer, 1984; Pedersen et al., 1990; Ueberschär, 1995). The LF group showed higher tryptic enzyme activity, especially from day 35 onwards. It can be assumed that either endogenous enzymes from the live feed could have positive effects on the fish larvae tryptic activity or the higher amount of nutrients ingested in LF fed groups. From 35 dph on, the degree of intestinal filling was over 90% in the LF group, whereas MD groups only reached values between 50 and 80% (not shown). The degree of intestinal filling seems to be particularly affected by the mechanical stimulation of the intestine by the feed. Liddle (2006) and Zeytin et al. (2016) showed that there is a correlation between intestinal filling level and trypsin secretion.

Liddle (2006); Zeytin et al. (2016) also showed that larger and bulkier feed particles have a stronger effect than smaller ones. This supports the explanation that the *Artemia nauplii* with a size of approximately 430µm stimulate the intestine more strongly than the MDs used in this experiment (between 35-40 dph size of 200-300µm, and from 40 dph onwards size of >300µm MD were used). In several other studies it has already been shown that the amount of trypsin correlates with the number of ingested nutrients (Pedersen et al., 1987; Ueberschär and Clemmesen, 1992; Ueberschär et al., 1992; Yúfera and Darias, 2007). Nevertheless, the degree of intestinal filling in our MD groups was still lower compared to live feed groups. Generally, lower intestinal filling in the feeding of MDs can also be attributed to the additional optical stimulus of swimming *Artemia nauplii*, which trigger a hunting reaction in the sea bass larvae rather than slowly sinking MDs (Lavens and Sorgeloos, 1996a). In order to compensate for this difference, MDs were fed at short intervals with an automatic feeder.

The dietary protein quantity did not appear to be directly related to the tryptic activity that was measured in this study. Protein content of fish protein meal depends on the raw materials but it contains at least 65 % dry matter proteins (Shaviklo, 2015). In this study, the fish protein concentration was 66% dry matter protein content. The used Calanus meal protein content was 55% dry matter. Based on their studies, Van Ballaer et al. (1985) noted that life feed (*Artemia*) contains an average protein content of 52% dry matter in nauplii and 56% dry matter in adults. However, the quality of dietary proteins or certain amino acids in microdiet may play an important role in regulating tryptic activity and pancreatic secretion in general (Tonheim et al., 2007). A sufficient supply of dietary amino acids is a prerequisite for high growth rates (Alam et al., 2013). Microdiets that contain proteins and other ingredients can be difficult to digest, especially since formulated diets are 60–90% dry matter while zooplankton are composed of only 10% dry matter (Kolkovski, 2001). A potential problem with the use of *Calanus finmarchicus* as a feed ingredient for fish larvae is the high degree of protein degradation post-mortem and a subsequent leaching of highly valuable nutrients when only frozen but not freeze-dried (Grabner et al., 1981).

In addition, a mixture of hydrolyzed and native protein appears to be a promising way to improve the MD. The former recipe with hydrolyzed protein (MDF) were found to be weak compared to new mixtures. The combination of native and hydrolyzed protein showed the best results. The addition of trypsin did not seem to offer any advantages since the trypsin used in these recipes is highly soluble in water and leaches fast but nevertheless larvae in MDC+T

groups showed higher growth rate. However, pancreatin appeared to be a more suitable candidate for further studies since it showed promising results in MDC+P in combination with the Calanus product. Thus, specifically in early larval phases, MDs could be used to bridge the critical phase of the sea bass when proper amounts of trypsin or pancreatin to be added to MD recipes were identified.

The replacement of live prey by formulated microdiets for larval feeding is of fundamental interest for the marine fish larvae rearing industry. A new MD formulation with a Calanus product (as an equivalent alternative to the live feeding by *Artemia Nauplii*) and the mixture of freeze-dried pancreas or trypsin have the potential to achieve better ingestion, digestion and survival of fish larvae. Further research is required to identify the proper combination of components as addressed in this research work and beyond, such as MD stability and leaching rates, before their true potential can be determined in marine fish larvae. With the results from these studies and the rapidly increasing knowledge in larval digestive physiology related to microparticulate feed and production techniques, replacement of live feed in early larval stages will become available soon.

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General Discussion

The ultimate goals of this PhD thesis were the optimization of feeding practices and formulated microdiets for marine fish larvae, in order to facilitate the replacement of live feeds (rotifers and *Artemia nauplii*) as first feed. Specifically, the study focused on the investigation of the larval digestive physiology and evaluated ontogenetic and nutritional deficiencies in the first feeding phase of gilthead sea bream (*Sparus aurata*) and European sea bass larvae (*Dicentrarchus labrax*). Accordingly, this thesis contributes to develop appropriate feeding protocols and presents for the first time a novel microdiet ingredient to enhance digestive capacity of marine fish larvae.

General consideration of methodological aspects in fish larval digestive physiology

Knowledge of digestive physiology of the larval stages under different feeding regimes has significant relevance to aquaculture. The effect of feeding practice or nutritional imbalance in relation to the development of the digestive system assessed by a number of indicators and used to characterize as "nutritional condition" of fish larvae (Lazo et al., 2011). Nutritional conditions were extensively reviewed by Ferron and Leggett (1994) and Catalán (2003) in terms of reliability, sensitivity, time response, size and age specificity, field versus laboratory estimates, processing time, costs, and requirements (Lazo et al., 2011). A great variety of morphometric, histological and biochemical variables have been routinely applied to investigate the digestive capacities and feed efficiency in fish larvae (Cara et al., 2007b). From a pool of different indicators, like the RNA:DNA ratio, C/N ratio, lipid profiles and the activity of some key enzymes involved in metabolism, Ferron and Leggett (1994) suggested that the activity of digestive enzymes can be a useful condition index due to their species and age specificity and sensitivity (Cara et al., 2007a).

The challenge of the above mentioned methods is not only restricted to the sensitive measurements of extremely small and delicate fish larvae, but also the determination of quick response to changes in different feeding practices (Rojas-García et al., 2001). Since we have evaluated short-term (hours- chapter I, II) and long-term (weeks- chapter III) changes of digestive capacity of fish larvae at different developmental stages under different feeding regimes, tryptic enzyme activity measurement was selected as an indicator and applied in all experiments in this study. Since marine fish larvae lack a stomach at first-feeding, trypsin represents the main proteolytic enzyme (Govoni et al., 1986). Therefore, tryptic enzyme activity in fish larvae seem to be an appropriate indicator of digestive capacity and can be used to

measure larval condition in response to changing environmental conditions such as the quality and quantity of ingested feed (Nolting et al., 1999).

The assessment of tryptic enzyme activity in individual larvae represents a major advancement to evaluate the individual response and variability compared to the low sensitivity of chromatographic methods, which rely on a large number of larvae in pooled samples (Gisbert et al., 2009; Suzer et al., 2013). Trypsin activity responds quickly to changes in nutritional status in the range from hours as can be seen in chapter 1 and 2, while RNA/DNA ratio, which is intensively used in other studies, responds slowly, usually after several days (Buckley et al., 1984). Findings from the previous studies (Navarro-Guillen et al., 2015; Tillner et al., 2013a; Tillner et al., 2014) showed, trypsin activity is found to be a suitable indicator for the nutritional studies and feeding activity of fish larvae. In addition, quantifying the changes of tryptic enzyme activity during feeding periods is found to be a suitable tool to optimize and adapt feeding regimes to the digestive capacity of marine fish larvae (Zeytin et al., 2016). This was confirmed by our first experiment in this thesis, where we evaluated the impact of different dietary regimes (rotifers, *Artemia nauplii* and microdiet) on the diurnal digestive tryptic enzyme activity at early stage gilthead sea bream (*Sparus aurata*) larvae (chapter I).

The amount of biomass required to perform reliable biochemical analyses has also been a constant constraint on gathering complete temporal series data during development (Rønnestad et al., 2013). Due to the small size of the animals and difficulties to determine feed intake and digestibility of microdiets, alternative methods and applications are applied in the development of successfully formulated microdiets (Conceição et al., 2007). In order to test whether it is possible to enhance the digestive capacity and consequently the tryptic enzyme activity in the gut of early stage of sea bass larvae, trigger substances were chosen and applied directly by the tube-feeding method in the present research (Chapter II). The technique chosen allowed us to determine if a given small volumes of dissolved trigger substance had an effect on the tryptic enzyme activity of an individual larva in a short-term experiment. Specifically, the effect of different substances that potentially enhance the trypsinogen production and secretion in various age stages of sea bass (*Dicentrarchus labrax*) larvae were investigated via tube-feeding method. At the time of the experimental planning, the aim was to identify various substances which may trigger the trypsinogen production in the early development stages of larvae. In order to generate new information about the optimal concentrations of trigger substances which can be supplemented into microdiets for marine fish larvae (Chapter III), tube feeding technique was

applied to create a matrix of potential ingredients. In chapter II, we used the tube-feeding study to draw limited conclusions based on the short-term experiment, while the real effects of promising supplements and ingredients were investigated in a long-term feeding experiment in chapter III. Furthermore, to ensure the required amount of trigger substances are added into larval gut through microdiet feeding, we set our tube-feeding experimental design to work with relatively developed larvae group (after 30 dph). Accordingly, the amount of trigger substances which we have added into microdiet in our follow-up experiment (Chapter III) was sufficient enough to trigger digestive capacity of fish larvae even after possible leaching problems. Hence, in this study we used tube-feeding to feed larvae between 30 and 60 dph and monitored their tryptic enzyme activity up to 10 hours after injection.

In general, the age ranges between 4 and 33 dph are used in tube-feeding experiments (Canada et al., 2016; Koven et al., 2002; Navarro-Guillén et al., 2014; Rønnestad et al., 2000; Tillner et al., 2013b). However, these studies were aimed mostly to demonstrate the effect of different dietary protein on feed intake, digestion, retention time or determine the response of digestive enzymes in first feeding fish larvae. In our study, we aimed to make a brief quantitative screening of the potential trigger substances to be used for a longer-term experiment. However, as it is discussed also in other studies (Conceição et al., 2007), data produced from tube-feeding experiments need very careful interpretation. On the one hand, tube-feeding experiments give an advantage to test the substances directly in individual larvae, but on the other hand the time is limiting to test all substances in the same experimental day. Accordingly, in our study, each potential trigger substance was tested in different days. According to our knowledge, several tube-feeding studies generally considered the measured parameter in relation to larval age (Canada et al., 2017; Tillner et al., 2013b). However, we suggest that measured parameters should take the length of larvae as a reference point for development stage instead of age for an easier interpretation. Finn et al. (2002) confirmed our observation that standard length reflects very well the developmental stage of fish larvae and represents a conservative reference.

The digestion process in early stages of fish larvae is known to be nutrient sensitive. Consequently, diet composition and feeding practices may delay or prevent larvae digestive development (Zambonino Infante and Cahu, 2001). In this regard, secretion rate of pancreatic enzymes is related to feed intake and nutrient composition (Rønnestad et al., 2007). Thus, reduced feed intake or an unbalanced microdiet may result in a decrease in secretion and, consequently reduced activity of trypsin (Pedersen et al., 1987; Ueberschär, 1995). Digestive

physiology and growth rates of fish larvae are closely related to each other. In practice, morphometric measurements (length and weight changes) and survival rate are also used as an indicator to evaluate the effect of diet composition and feeding regimes on the performance and intestinal development of early stage fish larvae (Betancor et al., 2012; Kolkovski et al., 2000; Suzer et al., 2011). In addition, morphological measurements are easy to realise, inexpensive and have low processing time (Catalán, 2003). Hence, in chapter III, the potential of a product sourced from freeze-dried Calanus meal as a major component, as well as trypsin and pancreatin as dietary enzyme supplements in microdiet for sea bass larvae were evaluated together with morphometric measurements (length and weight changes), survival rate and combination of biochemical method (trypsin activity).

With regard to constantly low trend of tryptic enzyme activity in starved control larvae groups compared to fed larvae groups, our study supports the suitability of measurement of tryptic enzyme activity as a tool for optimization of feeding practices (Chapter I). This hypothesis is line with the existence of digestion processes in relation to presence of feed input for feed ingestion in larvae of malabar grouper (Fujii et al., 2007) and sea bass (Tillner et al., 2014).

Microdiets versus live feeds

One of the goals of nutritional research on marine fish larvae is to generate knowledge that will enable us to replace live feed organisms with microdiet as early as possible in the larval stage. However, such a development is held back by a number of external and internal factors. Some studies (Kolkovski et al., 2000); Kolkovski et al. (2009); (Koven et al., 2001) indicate that the efficiency of utilization of feed particles by marine fish larvae is affected by chemical factors (feed attractants, free amino acids etc.), composition of microdiets (proteins, lipids, ingredients, moisture), ingestion (size, taste, shape), visual factors (colour, shape, movement), assimilation, absorption and digestion (digestive enzymes, digestive tract development, acid secretion). In addition, the tiny feed particles have an extremely high rate of leaching of water-soluble nutrients (Hamre et al., 2007). Although several commercial microdiets exist with relatively high potential for marine fish larvae during weaning period, additional limitations are faced when considering total replacement of live feeds from the onset of first feeding (Kolkovski et al., 2004). A diet particle needs to achieve a fine balance between leaching amino acids and other nutrients to act as feed attractant and digestibility of the particle to suit the undeveloped larvae digestive system (Burnell, 2013).

Certainly, the success of live feed replacement by microdiet depends on what degree larvae accept, digest and tolerate microdiets in order to reach an optimum growth and survival (Conceição et al., 2010b). In the present study, we have investigated microdiets versus live feeds in fish larvae nutrition from different aspects. Apart from the variation in the nutritional quality of the diet, Fernandezdiaz et al. (1994) and Kolkovski et al. (1997a) observed that different feeding regimes may have different physical (size, consistency) and chemical properties (attractiveness) that stimulates the variability of digestive enzyme secretion of fish larvae during the day. Therefore, the first assessment was to investigate whether there is any difference in diurnal digestive enzyme activity in gilthead sea bream larvae when fed with live feed (rotifer and Artemia) or microdiet (chapter 1). From a practical perspective, independently of their nutritional value, live feeds are easily detected and captured, due to their swimming movements in the water column, and highly digestible, given their lower nutrient concentration (water content >80%) (Conceição et al., 2010b). However, even the administration (live feeds three times a day per hand and microdiets every 15 minutes with an automatic feeding system) as well as nutritional profile of feeds were different, larvae at same age had the same pattern of tryptic enzyme activity during a day. In addition, independent of different feeding regimes, a third meal did not result in tryptic enzyme activities as high as after the first and second feeding, suggesting a limited proteolytic capacity in sea bream larvae to make use of a certain number of meals in a short time period. The same pattern of trypsin activity in larvae is probably modulated at the translational level by the dietary protein content in accordance with previous findings in sea bass larvae (Cahu et al., 2004) and southern flounder larvae (Alam et al., 2013). In chapter II, tube-feeding experiments showed that protein concentration had a positive effect on the tryptic activity in sea bass larvae (Zeytin et al., submitted). Therefore, in accordance to our results in diurnal tryptic enzyme activity under 24-hours culture condition (chapter 1), the optimal feeding time for sea bream larvae can be suggested to be scheduled from 07:00 a.m to 19:00 p.m between 25 and 45 dph. In addition, the number of meals could be reduced to two, instead of three. Nevertheless, further research on the impact of suggested feeding practice on the digestive physiology over a long-term growth study is needed.

The second assessment was to evaluate the replacement of live feed by newly formulated microdiets and to study the effect on growth performance, survival and tryptic enzyme activity in sea bass larvae. Zambonino Infante and Cahu (2001) suggested that digestive enzyme activities in early stages of marine fish larvae can be triggered by the nutritional composition of the diet. In

addition, Carneiro et al. (2003) noted that the low digestibility and nutritional quality of microdiets are factors that might explain their failure as a stand-alone starter feed. In our study, Calanus meal and pancreatin as a mixture of various enzymes and trypsin found to be appropriate for effectively replacement of fish meal and eventually live feed in sea bass larvae diets to achieve higher growth, tryptic enzyme activity, and survival rates (chapter III). Marine copepods and their nauplii stages are considered as the best feed source for marine fish larvae (Conceição et al., 2010b). Live feeds ingested by larvae contain exogenous substances such as gut neuropeptides, enzymes and nutritional growth factors that contribute to the digestion of prey, frequently omitted in microdiets (Rosenlund et al., 1997). Therefore, in addition to Calanus meal, supplemented proteolytic enzymes seemed to bridge the rather poor digestive enzyme capacity in the early stages of sea bass larvae.

Composition of experimental microdiets in chapter III were in accordance with most commercial used microdiets in sea bass and sea bream hatcheries. In our study, fish meal based diet had 64% (MDF), Calanus meal based diet had 57% (MDC) crude protein, 11% and 19% crude lipid, respectively. The average values for commercial microdiets are 62% crude protein and 17% crude lipid, and where the main ingredients are fish meal, squid meal, shrimp meal, wheat gluten, fish solubles, fish oil and soy lecithin (Cahu and Zambonino Infante, 2001; Seiliez et al., 2006; Villeneuve et al., 2005). In comparison to microdiets, average composition of *Artemia nauplii* and adults have in average 52 to 56% crude protein, 18 to 11% lipid in dry matter, respectively (based on the evaluation of many studies reviewed by Leger et. al. 1986). According to the results of chapter III, no significant differences of growth performance were found in larvae fed with live feed and Calanus based diets. However, tryptic enzyme activity was significantly higher in the live feed group to other feeding groups. Liddle (2006) and Zeytin et al. (2016) (chapter I) showed that there is a correlation between intestinal filling level and trypsin secretion. These mentioned studies also showed that larger and bulkier feed particles have a stronger effect than smaller ones. This was also confirmed in chapter III as the degree of intestinal filling was over 90% in the LF group, whereas MD groups only reached values between 50 and 80% (data not shown). These results suggest that the experimental microdiets have the potential of decreasing or replacing live feeds in early stages in sea bass larvae nutrition. In this thesis, Chapter III showed that it is possible to improve larval development through more nutritional microdiets, however more studies are necessary to understand how Calanus meal based diets act on the digestive physiology of fish larvae.

A novel microdiet ingredients to enhance digestive capacity of marine fish larvae

Commercially available fish diets are produced from a large variety of feed ingredients (Jobling et al., 2001). Different ingredients have different nutritional and physical properties (Hertrampf and Piedad-Pascual, 2000). A ingredient should contribute to the nutritional value of the feed (amino acids, fatty acids, vitamins and minerals, its digestibility and palatability), and improve the physical integrity of the feed (pellet hardness, durability, binding, density, pellet water stability) (Francis et al., 2001; Hamre et al., 2013; Tusche et al., 2011). For rearing marine fish larvae, it is important to consider the physiology and ontogeny of the larval digestive system, feeding regime, technology as well as each species nutritional requirements in the process of microdiets preparation (Saleh et al., 2013).

It is still a major bottleneck to have successful use of microdiets from first-feeding. The current problem of microdiet is still associated with nutritional deficiencies, nutrient leaching, low digestibility and acceptability which reduces feed intake (Baskerville-Bridges and Kling, 2000a). In addition, during feed production, the physical and functional characteristics of ingredients can cause considerable variation in the quality of produced microdiet (Yufera et al., 2016). Protein is the main component in diets and a growth-limiting nutrient (Gonzales, 2013). While fish meal is well recognised as protein source for juvenile and adult fish (Jobling et al., 2001; Kaushik and Seiliez, 2010), its proteins may be too complex for easy digestion in fish larvae (Kolkovski, 2001). Moreover, there is a need to identify replacements for fish meal such as plant or marine protein sources (Hertrampf and Piedad-Pascual, 2000). However, plant protein riched diets may be deficient in some micronutrients present in marine ingredients or contain antinutritional factors. Beside its digestibility, the composition of nutrients in microdiets need to be attractive to the larvae. Since marine copepods and their nauplii stages are considered as the best feed source for marine fish larvae, the final experiment of this thesis (chapter III) focused to examine the effect of a product sourced from freeze-dried Calanus meal as a major protein component. The aim was also to further develop and improve the quality of already existing commercial microdiets and achieve equivalent results compared to traditionally used live feeds. In addition, trypsin and pancreatin as digestive enzyme were added as supplements in microdiets to enhance digestive capacity, growth and survival of sea bass larvae. Experiments to investigate the effects of the addition of enzyme-like active ingredients to fish feed have been carried out by Buchanan et al. (1997); (Dabrowski and Glogowski, 1977; Divakaran and Velasco, 1999; González-Zamorano et al., 2013). However, results indicate that growth was not enhanced by the

inclusion of digestive enzymes in these feeds (Dabrowski and Glogowski, 1977; González-Zamorano et al., 2013). Nevertheless, in our study, the combination of Calanus meal based diet and digestive enzyme supplements turned out to contribute substantially to digestive capacity, growth and survival of sea bass larvae.

Certainly, feed costs are a major consideration in aquaculture that can account for 40 to 60% of total production costs. The freeze-dried Calanus meal used in this study costed 156 Euro/kg (personal communication with MiniPro A.S.). However, the cost of microdiet is negligible in terms of amount used compared to diets for adults. On the other hand, the current use of live feed (rotifers and *Artemia*) for larviculture is a labor-intensive, time-consuming process and costly. Therefore, a nutritive microdiet should be formulated for a reliable and safe production of fish larvae. Furthermore, microdiet feeding via hand leads negative impact on water quality. In most cases, feed rations are higher than necessary, and this causes negative impact on water quality. However, a professional automatic feeding system can create small amounts of feed administration in short times and could reduce the deposition of feed rest on the bottom of the tanks (Koven et al., 2001).

In order to gain accurate information for optimisation of microdiets and feeding practices for real hatchery management, research studies must reflect the same environmental condition in their experimental design for an accurate information. In this PhD thesis, microdiet feeding in chapter I and III was administered via an automatic microdiet dispenser system (AMD-system®) which allowed administering microdiets periodically in adjusted quantities by the number of shots per feeding event. Feeding frequencies and amounts of microdiets with number of shots were adjusted according to larval age and experimental microdiets were present in the water column most of the time during the feeding period. Beside the feeding system, microdiet production needs also professionals and experts to set necessary techniques and methods for manufacturing microdiet particles. In this study, the production of the novel experimental microdiets used in Chapter III were manufactured by the Norwegian company, MiniPro A/S. Therefore, the reliable results gained in this study permits further knowledge and understanding of nutritional requirements of marine fish larvae and finally overcome the lack of digestive capacity problems with new microdiet components in the early developmental stages.

In summary, it can be concluded that for the advancement of feeding practices in larviculture, it is important to know how and when the larvae feed and digest different feeding regimes. In addition, dietary proteolytic enzymes were added in order to bridge the rather poor

digestive enzyme capacity in the early stages of sea bass larvae and compared to traditional live feed (*Artemia nauplii*) and a standard micro diet formulation. These results could contribute to determining suitable feeding schedules with a proper quantity in the early stages of marine fish larvae to maximize nutrient utilization and growth.

Further research needs

The achieved quality of the microdiets in this study can be suitable for marine larvae and could be classified as ready for commercial production. Although the results from the evaluation of the new formulations are very promising, there is still potential for further improvements. In particular, it seems that follow-up trials will be needed to identify the optimal concentrations of digestive enzymes as supplements in the microdiet industry. In addition, since there is a close relationship between larval nutrition at first feeding and skeletal abnormalities, future research should focus in identifying the relationship and if there is an effect when using microdiets as first feed in larvae.

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Summary

Global consumption of seafood continues to increase and consequently, the farming of marine species has become a rapidly growing industry. This rapid growth of marine aquaculture industry requires fry with a reliable quality and quantity. Despite considerable progress in aquaculture, high mortalities during larval production is still considered as a bottleneck for aquaculture production. These problems of survival and quality in larval production are apparently related to the performance of the digestive physiology during ontogeny.

In this critical period, larval digestion takes place in the gut, in which trypsin functions as a key proteolytic enzyme. Considering the low digestive capacity during these developmental changes, this thesis used the observation of tryptic enzyme activity during feeding periods as an indicator to optimize and adapt feeding practices and feed components to the digestive capacity of the sea bass and sea bream during larval development. Accordingly, optimisations towards fish larval nutrition were studied to facilitate the replacement of live feeds (rotifers and Artemia nauplii) as first feed. The first experiment in this thesis evaluated the impact of different dietary treatments on the diurnal digestive tryptic enzyme activity in gilthead sea bream (*Sparus aurata*) larvae. In order to evaluate optimum feeding time and frequency, several short-term experiments were conducted at different ages with different dietary treatments such as traditionally used live feeds (rotifers and *Artemia*) and microdiet. The results suggested that no matter what kind of diet was applied, sea bream larvae have a limited digestive capacity at some point in time during the day. The second study investigated the effect of different substances that potentially can enhance the trypsinogen production and secretion in various age stages of sea bass (*Dicentrarchus labrax*) larvae via tube-feeding method. The substances under evaluation were selected as potential ingredients to be used in the formulation of microdiets for marine fish larvae. The results of this experiment provided a useful matrix used in the next experiment to identify the relative advantages of the various ingredients, their concentration and their use at specific life stage of marine fish larvae. Specifically, an increase in tryptic enzyme activity after injection of phytohaemagglutinin, protein concentration, Calanus meal *(Calanus finmarchicus)* and the extract of the commercial microdiet MiniPro in sea bass larvae supported the hypothesis that selected substances can trigger the release of trypsinogen beyond the "normal" rate at specific developmental stage. However, it was also demonstrated that the concentration of the injected substances has a significant impact on the results. The final study of this thesis focused on the potential of a product sourced from freeze-dried Calanus meal as a major component, as well as

trypsin and pancreatin as digestive enzyme supplements in microdiet for sea bass larvae. The main goal of this experiment was to compare and evaluate the ontogenetic development of tryptic enzyme activity in larval sea bass fed either live feed, a commercial microdiet or the microdiet containing the Calanus meal as major ingredient. Results suggested that Calanus meal and pancreatin as a mixture of various enzymes and trypsin could effectively replace fish meal in sea bass larvae diets to achieve higher growth, tryptic enzyme activity, and survival rates.

Zusammenfassung

Trotz beachtlicher Entwicklungen im Bereich der Aquakultur, stellt die hohe Mortalität in der Larvenproduktion immer noch den entscheidenden Engpass dar. Die Probleme durch unzureichende Überlebensraten und Qualität in der Larvenproduktion kann eindeutig auf die Verdauungsleistung während der Larvenentwicklung zurückgeführt werden. In dieser kritischen Phase des Larvenstadiums findet die Verdauung im Darm statt, wobei Trypsin als Schlüsselenzym fungiert.

Unter Berücksichtigung der geringen Verdauungskapazität während der larvalen Entwicklungsphase wurde in der vorliegenden Dissertation, die Trypsinaktivität von Fischlarven evaluiert, um Fütterungspraktiken zu optimieren sowie Futterkomponenten an die Verdauungskapazität anzupassen. Dementsprechend wurde im ersten Experiment dieser Arbeit der Einfluss von unterschiedlichen Futterverabreichungen auf den Tagesrhythmus der Trypsinaktivität von Goldbrassen-Larven (*Sparus aurata*) untersucht. Um die optimale Fütterungszeit und Häufigkeit der Fütterung zu bestimmen, wurden mehrere Kurzzeit-Experimente mit unterschiedlichen Larvenstadien und unterschiedlichen Futterverabreichungen, wie z.B. traditionell genutzte Lebendfutterorganismen (Rotatorien und *Artemien*) oder sogenannte Microdiets, durchgeführt. Die Ergebnisse zeigten, dass unabhängig von der Nahrung Goldbrassenlarven an gewissen Zeitpunkten nur eingeschränkte Verdauungskapazitäten aufweisen. Die zweite Studie dieser Thesis diente dazu, mithilfe der sogenannten tube-feeding Methode die Auswirkungen von verschiedenen Substanzen, die potentziell die Produktion sowie Ausschüttung von Trypsinogen fördern, auf unterschiedliche Altersstadien von Wolfsbarschlarven (*Dicentrarchus labrax*) zu untersuchen. Die untersuchten Futterbestandteile wurden ausgewählt, um als potenzielle Inhaltsstoffe für Microdiets zu dienen. Besonders der Anstieg in Trypsinaktivität nach der Injektion von Phytohämagglutinin, Fischproteinkonzentrat, Calanus-Mehl (*Calanus finmarchicus*) und einem Extrakt der kommerziell verwendeten Microdiet MiniPro unterstützten die Hypothese, das ausgesuchte Substanzen die Ausschüttung von Trypsinogen über die übliche Rate von Wolfsbarschlarven hinaus fördern kann. Allerdings wurde auch deutlich, dass die Menge der injizierten Substanzen einen entscheidenden Einfluss auf die Ergebnisse hat. Darauf aufbauend wurde in der abschließenden Studie das Potenzial eines gefriergetrockneten Calanus-Mehls, Fischmehls sowie Trypsin und Pankreatin als Supplemente in Microdiets für Wolfsbarschlarven evaluiert. Das Ziel dieses Experiments war, die ontogentische Entwicklung der Trypsinaktivität von Wolfsbarschlarven bei Einsatz dieser

unterschiedlichen Futtermittel zu evaluieren. Die Ergebnisse deuten darauf hin, dass Fischmehl im Futter für Wolfsbarschlarven effektiv durch Calanus-Mehl und Pankreatin- und Trypsin-Supplementation ersetzt werden kann, um höheres Wachstum, optimierte Trypsinaktivitäten und Überlebensraten zu erreichen.

APPENDIX

Tube-feeding technique to assess trigger substances on the tryptic enzyme activity

A method of controlled tube-feeding developed by (Rust et al., 1993) encouraged to overcome some of the limitations in nutritional studies with fish larvae. Tube-feeding method enables the monitoring of the gut passage and ensures the tested substance is administrated exactly in the gut of fish larvae because of the visual control of transparent gut areas (Fig.A-1). The method has been modified by Rønnestad et al. (2001) and permits more precise estimate of the performance of the digestive system.

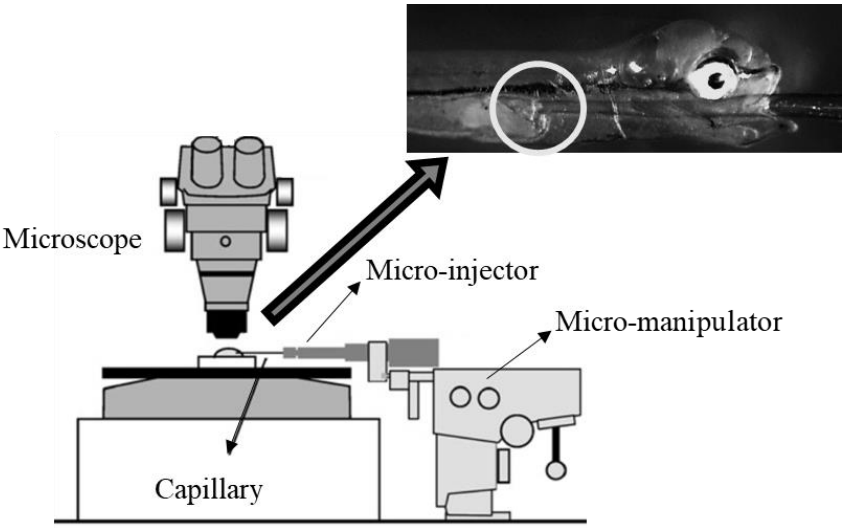

Fig. A-1. Schematic drawing of the equipment set-up for tube-feeding of sea bass larvae via micro-injection (Rønnestad et al., 2001).

The setup comprises a stereo-dissecting microscope with micro-manipulator. A nanoliter injector (Nanoliter 2000, WORLD PRECISION INSTRUMENTS) is attached to the micromanipulator. A handmade glass capillary is adapted to the mouth and oesophagus diameter of small larvae and fastened to the nanoliter injector. To avoid causing damage to the gut epithelium, the capillary tip is polished to a conical shape. For larger larvae, a plastic capillary tube (Sigma, St. Louis, MO, USA) with inner diameter of 0.19 or 0.32 mm, depending on larval size, is used. No special polishing is required for these plastic capillary tubes (Rønnestad et al., 2001).

Tube-feeding was performed on groups of randomly selected larvae. Larvae were removed individually from the tanks by a pipette with wide opening and before injection, the larvae anaesthetised using MS-222 (Tricaine methanesulfonate, SIGMA-ALDRICH) and then

gently placed on a microscopic slide in a droplet of clean seawater (Fig.A-2). The concentration and time in the anaesthetic bath are adjusted for each individual, typically 30 µg/ml and 1–5 min. The importance is to maintain continuous opercular movements to ensure survival (Rust et al., 1993), and a rapid recovery to ensure proper physiological function of the digestive system. After injection, each larva was rinsed with clean seawater and transferred to an incubation well within a 6-well multidish. The larvae remained in the incubation well until sampling (0,5h, 1h, 2h, 4h, 8h, 10h, depending on the substance injected). Each larva was then gently transferred to an Eppendorf tube with one droplet of seawater and frozen at -80°C, in order to interrupt any enzymatic activity.

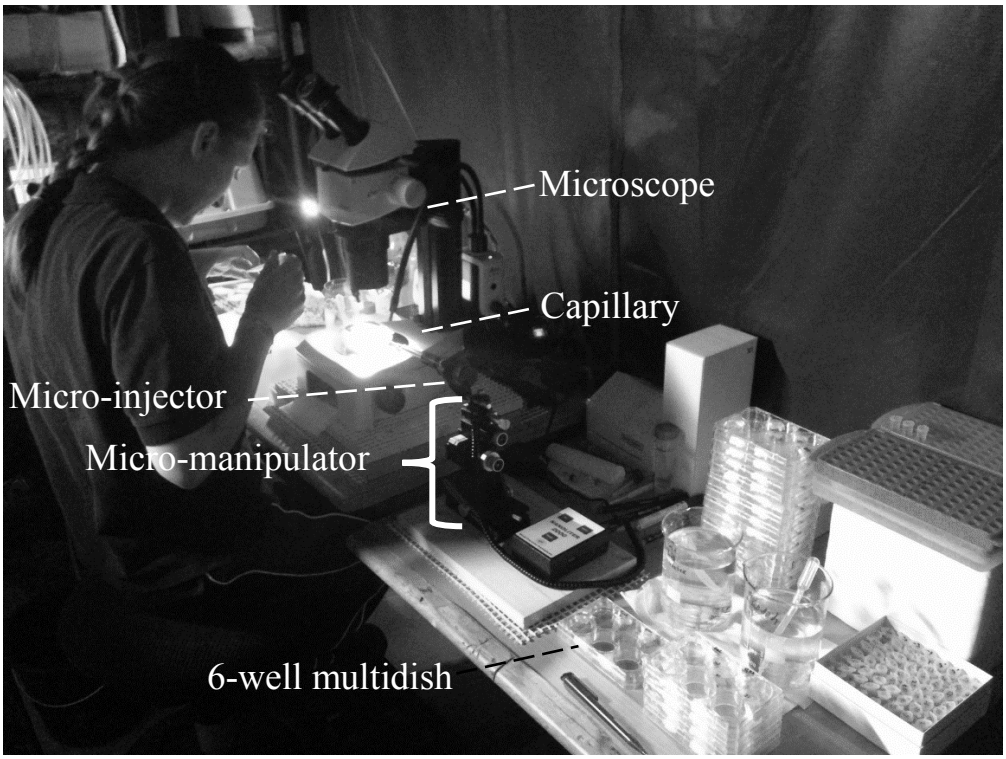

Fig.A-2. Experimental tube-feeding set-up next to larval rearing tanks (chapter II).

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