Aquaculture 457 (2016) 85–90

Contents lists available at ScienceDirect

Aquaculture

journal homepage: www.elsevier.com/locate/aquaculture

Short Communication

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

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ARTICLE INFO

*Article history:*
Received 30 November 2015
Received in revised form 27 January 2016
Accepted 11 February 2016
Available online 12 February 2016

*Keywords:*
Sea bream larvae
*Sparus aurata*
Trypsin
Diurnal rhythm
Digestive capacity

ABSTRACT

Knowledge about the dynamics of the diurnal digestive enzyme capacity during early larval stages of fish is crucial for the determination of appropriate feeding time and frequency. However, there is little data available considering only few species of fish. 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 (group fed with rotifers *Brachionus plicatilis* (R), with *Artemia* (A), with rotifers and *Artemia* (RA) and 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 min with an automatic feeding system. In addition, for each experiment, a subgroup of larvae deprived of food was 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 at 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 groups A and MD at 44 dph showed a similar diurnal pattern in tryptic enzyme activity although group MD was 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.
*Statement of relevance:* This study will help to reduce the cost for fry production.


## 1. Introduction

It is a common knowledge that under natural conditions a circadian rhythm in feed intake exists in the larval and early juvenile stages of fish. This metabolic rhythm is linked to the environmental changes such as illumination, temperature, or food availability. (Boujard and Leatherland, 1992; Ueberschär, 1995). 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 in many species, feeding activity specifically occurs at sunrise and sunset (Kane, 1984; Ueberschär, 1995). However, in intensive culture conditions of hatcheries, larvae production is predominantly carried out under continuous illumination and frequent feeding. Under such conditions the larvae will capture prey continuously. For instance, gilthead sea bream larvae fed continuously without apparent satiation during the availability of food (Rønnestad et al., 2013). Studies indicate that most marine fish larvae lack a morphological and functional stomach (Govoni et al., 1986), thus limiting the digestive enzyme capacity to digest ingested food. Accordingly, it is still argued, whether continuous feeding has benefits for growth and survival of larvae; or rather overstrains the digestive capacity of larvae.

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), usually in short term experiments. For example, Drossou et al. (2006) determined the effects of two test diets on tryptic activity during early stages of Nile tilapia (*Oreochromis niloticus*). Similarly, Tillner et al. (2013) evaluated the daily pattern of tryptic enzyme activity of Atlantic cod larvae (*Gadus morhua*) under 1 meal or 2 meal feeding protocol. Recently, Navarro-Guillen et al. (2015) examined diel food intake and tryptic enzyme activity of Senegalese sole larvae under laboratory conditions. These studies investigated the mealtime, gut fullness, and prey density under different light conditions on digestion (Fujii et al., 2007; MacKenzie et al., 1999; Navarro-Guillen et al., 2015; Tillner et al., 2013), but did not focus on the diurnal pattern of digestive enzymes under different feeding regimes.

Although considerable improvements in the quality of formulated diets were achieved in the last decades (Cahu et al., 2003; Hamre et al., 2013), current feeding protocols of marine fish larvae still depend on live prey, especially in the first-feeding stages and early larval stages

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http://dx.doi.org/10.1016/j.aquaculture.2016.02.017
0044-8486/

(Conceição et al., 2010; Drossou et al., 2006; Holt et al., 2011). Several studies have been evaluating the feeding behavior and digestive physiology of sea bream larvae with regard to MicroDiet ingestion (Fernandezdiaz et al., 1994; Kolkovski et al., 1997; Parra and Yúfera, 2000). Nevertheless, there is little and inconclusive data available to determine the optimum feeding time and frequency for the different developmental stages of larvae under a feeding regime applying to MicroDiet. Accordingly, the aims of this study were 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 variation among larvae fed with live and formulated feed. Therefore, several short-term experiments were conducted at different stages of development with various 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 lx 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 5 μm) and treated with UV light (55 W UVC), and circulated among the big tank and the 12 experimental tanks (Fig. 1A). 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, 800,000 cells/mL).

### 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 $mL^{-1}$, and 15 $mL^{-1}$ respectively. Group RA; sampled at 26 dph, was fed with rotifers and *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, was fed with *Artemia* nauplii at densities of 4 $mL^{-1}$. Groups with live feed were fed three times a day at 07:15, 14:15 and 22:15 o'clock. Group MD, sampled at 26 and 44 dph, was fed with MicroDiet (Skretting, Belgium) every 15 min 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 15 cm) 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 had water exchange during the experiment. Six tanks were assigned to each of the following groups (R, RA, A and MD) (see Fig. 1A). Larvae from each replicate tank (total n = 6) representing each dietary treatment were collected every full hour and were transferred into an Eppendorf vial between 06:30–01:30 o'clock and immediately stored at −80 °C until analysis in order to prevent 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 a microscope using a millimetric scale (Fig. 1B). Each individual was transferred into a 1.5 mL Eppendorf vial and homogenized in ice-cold Tris-Buffer (TRIS–HCl, 0.1 M, pH 8, MERCK, Art. no. 1083820500, including $CaCl_2 \times 2H_2O$, 0.02 M, MERCK, Art. no. 2382) by using a motorized pestle with a 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 125 μL or 250 μL; 44 dph larvae with 500 μL). The homogenate was centrifuged for 45 min (4110 × *g* 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 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 tryptic enzyme activity measurement in order to avoid any proteolytic activity. Tryptic activity was expressed as the amount of hydrolysed substrate (μmol MCA) per minute and larva.

### 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 heteroscedastic due to the different levels of feeding groups, age and sampling event. These assumptions were 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 (groups 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, (Hasler, 2014). The results were 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, $p < 0.05$).

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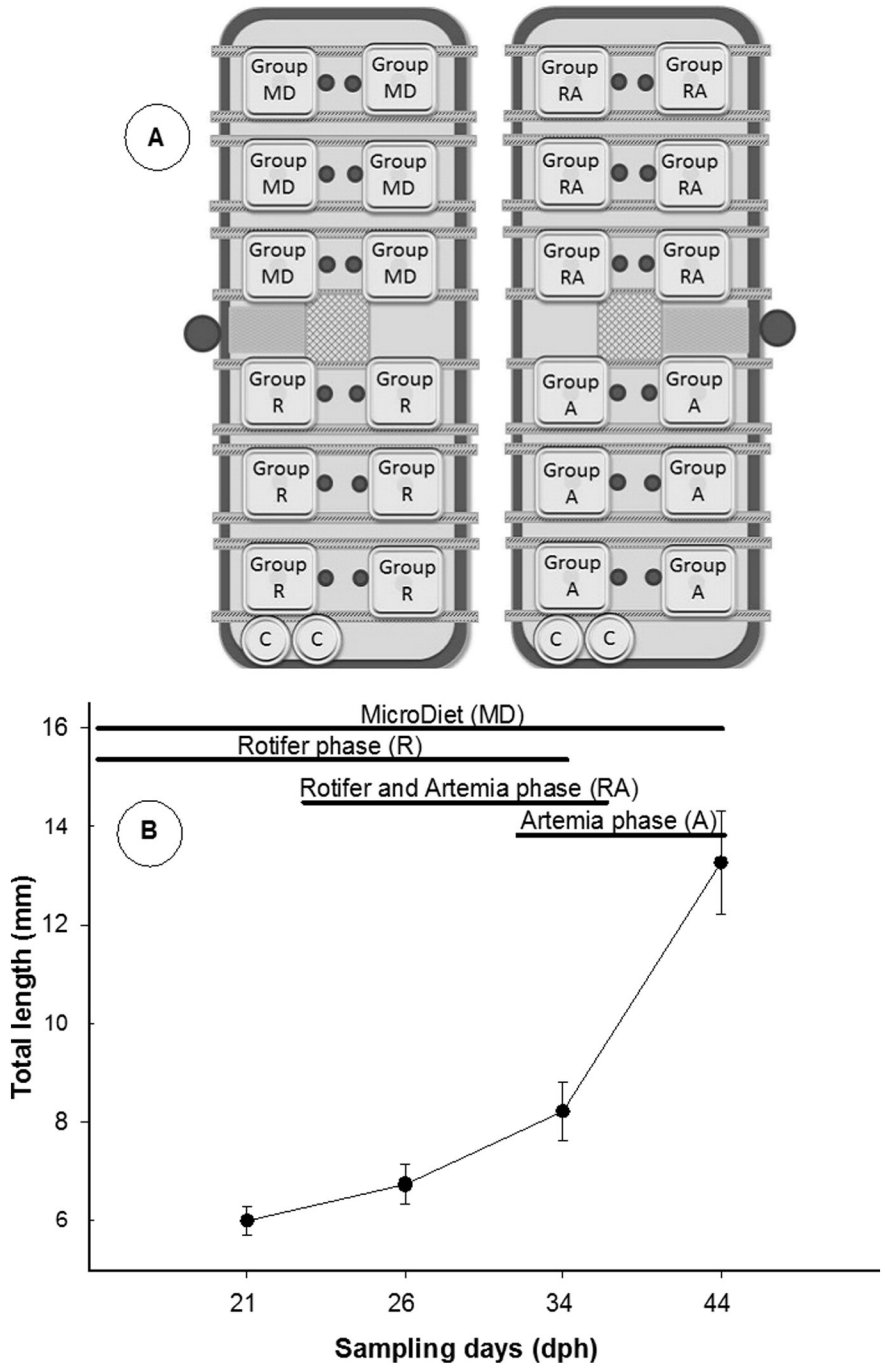


**Fig. 1.** (A) Schematics of the experimental setup. (B) Feeding regime of different groups and total length of sampled sea bream larvae over the experimental period of 44 days. Data are presented as mean ± S.D. (n = 120 at 21 dph, n = 360 at 26 dph, n = 120 at 34 dph, n = 240 at 44 dph).

### *3.2. Group 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. 2. 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. 2A) the diurnal pattern of tryptic enzyme activity showed various peaks and drops between each sampling event. 1 h after the second feeding, the tryptic enzyme activity depicted 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. 2B) 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 the second feeding event. At 34 dph (Fig. 2C) a clear difference between fed and control larvae can be observed regarding the enzyme activity pattern throughout the sampling period of 20 h.

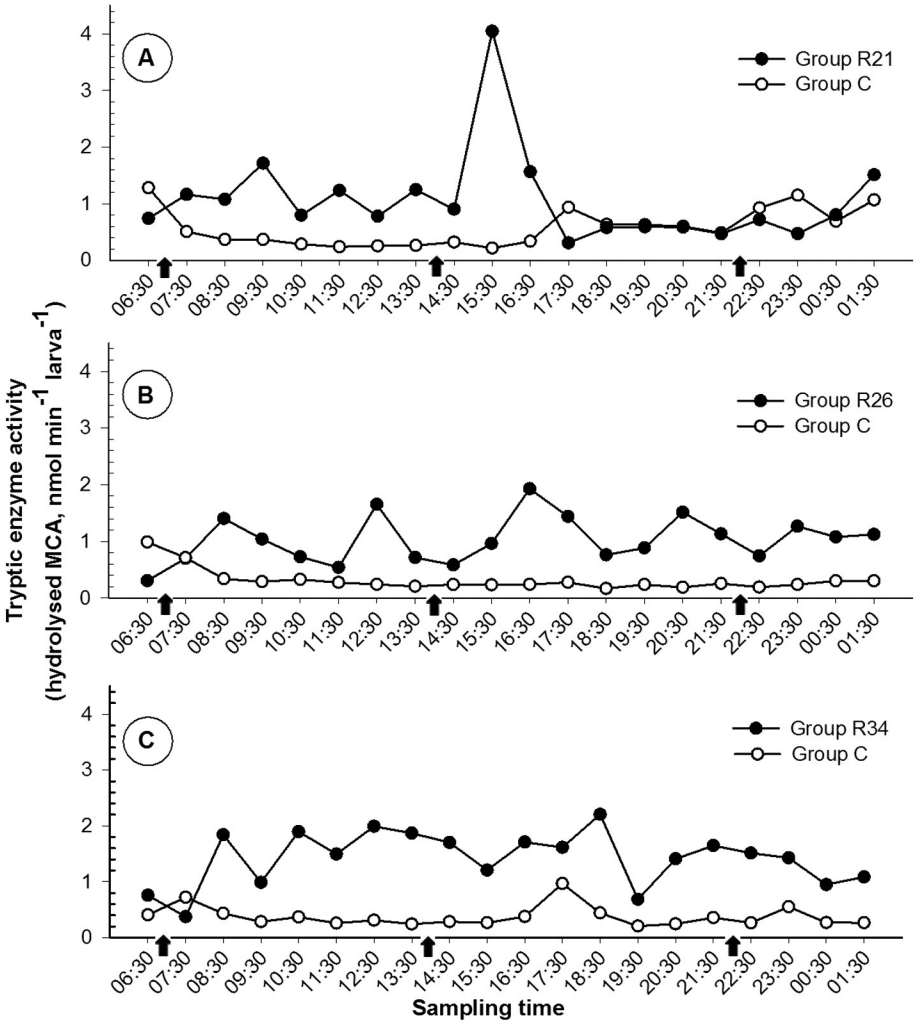


**Fig. 2.** Diurnal pattern of tryptic enzyme activity (nmol MCA $min^{-1}$ $larva^{-1}$) levels of fed (—●—) and control (—○—) sea bream larvae at three different age groups (A) 21 dph, (B) 26 dph, and (C) 34 dph. Sampling of fed and control larvae took place between 06:30 to 01:30 every full hour under 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. 3A. 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. 3B) 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 was observed at 13:30, 14:30 and 22:30 where significant differences in activity were detected in relation to other sampling events (mult comp test, $p < 0.05$).

### 3.5. Group fed with MicroDiet

Two different age groups (26 and 44 dph) of sea bream larvae were continuously fed with MicroDiet 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. 3A and 3B). There was a significant difference in lengths between 26 dph and 44 dph of larvae and the mean tryptic enzyme activity showed significant differences at 12:30, 18.30 and 20:30 (data not shown) (mult comp test, $p < 0.05$).

## 4. Discussion

This study showed that there exist impacts on the diurnal tryptic enzyme activity of sea bream larvae due to different dietary treatments at various stages of development. Based on an hourly monitoring of tryptic enzyme activity over 20 h, it was found that the groups fed with *Artemia* and MicroDiet showed a clear diurnal pattern in tryptic enzyme activity in sea bream larvae at different ages. However, groups fed with rotifers showed less pronounced effect in the early stages. These various patterns in tryptic enzyme activity are clearly linked to the kind of feeding regimes. In general, the tryptic enzyme activity was significantly lower in groups fed with rotifers (groups R21, R26 and R34, Fig. 2A, B, C) as compared to groups fed with *Artemia* and MicroDiet (groups RA26, MD26, A44 and MD44, Fig. 3A, B). This result is in line with the study

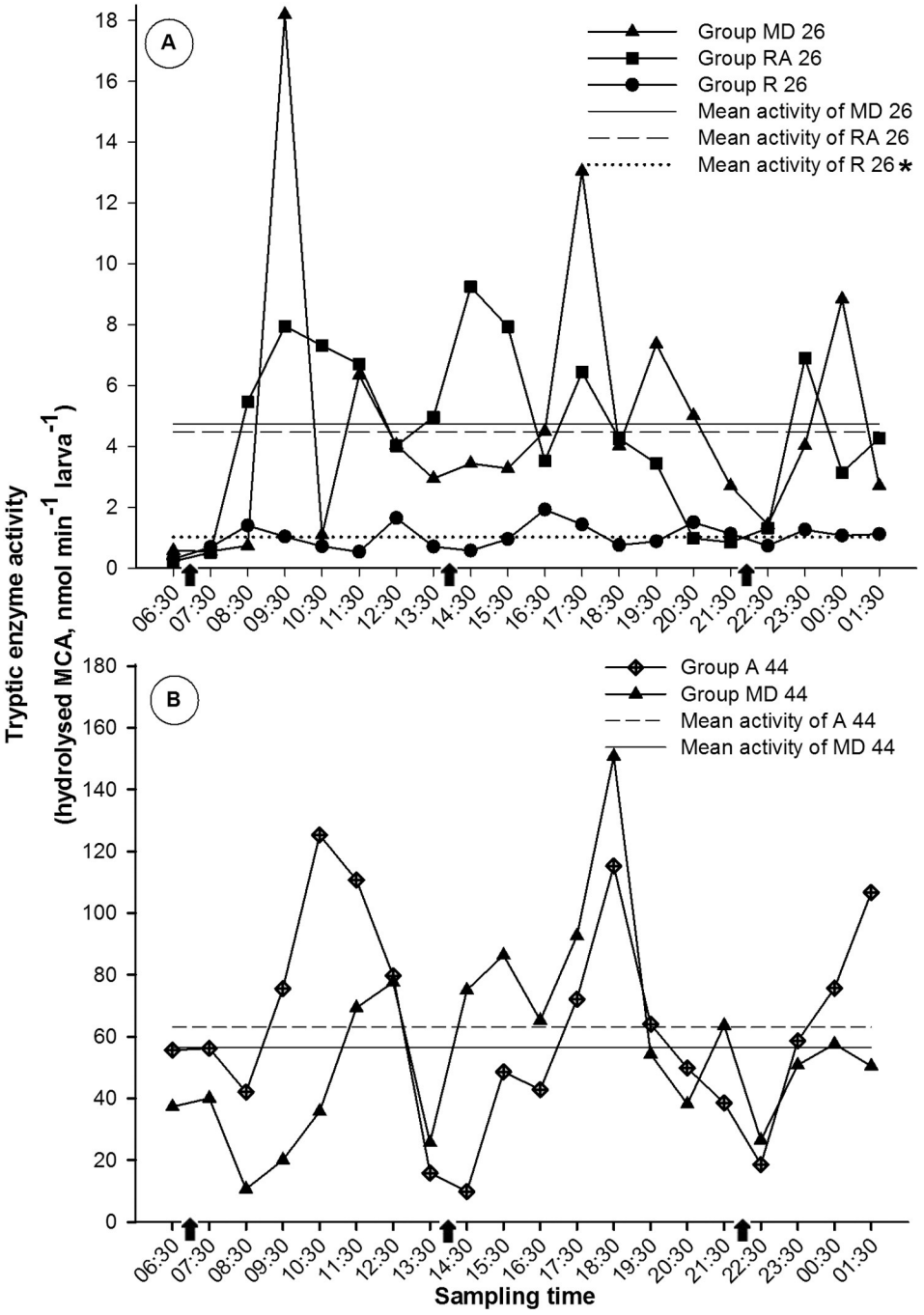


**Fig. 3.** (A) Diurnal pattern of tryptic enzyme activity (nmol MCA $min^{-1}$ $larva^{-1}$) of rotifer fed; group R (—○—), rotifer and *Artemia* fed; group RA (—■—) and MicroDiet fed; group MD (—▲—) sea bream larvae at 26 dph. (B) Diurnal pattern of tryptic enzyme activity (nmol MCA $min^{-1}$ $larva^{-1}$) 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 groups MD26 and MD44 respectively. Black arrows indicate the administration of rotifers and *Artemia* three times a day, where MicroDiet group was fed every 15 min. Asterisk indicates significant differences between group R26 and; group RA 26 and group MD 26 in the overall experiment. No symbols indicate no significant differences (mult comp test, $p < 0.05$).

by Gamboa-Delgado et al. (2011), who demonstrated that tryptic activity in Senegalese sole larvae fed with rotifers showed lower activities compared to larvae fed with *Artemia* and formulated diets. In addition, in our experiments, the groups fed with rotifers had lower gut fullness (between 25 to 50%) compared to groups fed with *Artemia* and MicroDiet (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 (Liddle, 2006). The mechanical stimulation in the gut is supposed to depend on the size of the food particles (more "bulky" food particles induce likely a stronger stimulation). Accordingly, the type of food particle ingested (with *Artemia* and MicroDiet, the larvae apparently ingest more particles in a short period of time as compared to rotifers) might result in a different degree of gut fullness. This could be the reason for the small fluctuations in group R, whereas more pronounced peaks were observed after meal time in groups 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" of digesting the food

and/or trigger substances from the food which cause a different reaction in trypsinogen secretion. Recent tube-feeding experiments showed that some natural products which contain a high level of protein concentration had a positive effect on the tryptic activity in sea bass larvae (Zeytin et al., in preparation). On the other hand, a third meal of rotifers and *Artemia* did not result in tryptic enzyme activities as high as after the first and second feeding events. This may indicate limited proteolytic capacity in sea bream larvae that prevents to benefit of a continuous supply of feed in a short time period. These findings are line with Tillner et al. (2014), who pointed out that a reduction in tryptic enzyme activity after several feeding events indicate a limited diurnal digestive capacity in sea bass larvae. The degree to which fish larvae were able to accommodate feed in the gut and to digest it efficiently might be dependent 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 and in the investigated developmental stages. Nevertheless, although the reaction of the third meal was rather weak compared to the previous feeding events, the mean tryptic enzyme activity value among fed and control groups throughout the experimental period was significantly different (mult comp test, $p < 0.05$) (Fig. 2A, B, C). This low activity value in the control group demonstrated the significance of a mechanical stimulation of the digestive tract as consequences of the ingestion of feed.

The comparison of the tryptic activity in groups A and MD at 44 dph showed a quite similar diurnal pattern in tryptic enzyme activity although group MD was continuously fed with MicroDiet (Fig. 3B). The mean tryptic enzyme activity of groups A and MD (as mean value with 95% confidence limit) was very similar with no significant differences, suggesting that both groups do invest the similar amount of trypsinogen to digest the different diets. In contrast to the fed groups, tryptic enzyme activity remained significantly lower in group C with larvae deprived of food throughout the entire sampling period. This confirmed 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 groups under different feeding regimes (between R26, RA26, MD26 and A44, MD44).

In summary, this study demonstrated that a limited digestive capacity in sea bream larvae exist at some point in time during the day, with all diets used in this study. The study also suggested that early larval stages might not be able to digest the applied feed over 24 h with constant efficiency. In general, the results of this study 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 in hatcheries. This could be especially important to minimize deterioration of the water quality by optimizing the feeding events when using MicroDiet in sea bream hatcheries.

Based on the results in this study, further research is important focusing on optimization of feeding protocols, where feeding is being reduced in the afternoon and evening and compared with continuous food supply under 24 h illumination. Furthermore, other major enzymes than trypsin that contribute to digestion of fish in early stages, such as pepsin, amylase, and lipases should be considered in future investigations, in order to get accurate and comprehensive results.

## Acknowledgments

This work was funded by the DBU — Deutsche Bundesstiftung Umwelt under grant agreement no. AZ 30586. The authors wish to thank Dr. Robert Tillner for his assistance during larval rearing.

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