Determination of the inhibitory effects of microdiets used in routine commercial feeding protocols on protease activities of *Argyrosomus regius* (Asso, 1801) larva

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Abstract

The aim of this study was to determine the inhibitory effects of feed ingredients on protease activities of *Argyrosomus regius* larvae using *in vitro* techniques. *A. regius* larvae fed on a commercial feeding procedure were sampled thirteen times, during the sampling period (from 3 to 32 days after hatching (DAH)). The differences observed in protease activities of meagre larvae during the sampling period were statistically significant ($p<0.05$). The lowest and highest protease activities of meagre larvae were 5.95±0.6 U/mg protein (15 DAH) and 211.21±12.56 U/mg protein (7 DAH), respectively. The fluctuations observed in protease activities of *A. regius* larvae were between 10 DAH and 32 DAH. Commercial diets such as Orange Start-S (100-200µ), Orange Start-L (200-300µ), Orange Nurse-XS (300-500µ), Orange Grow-S (300-500µ) and Orange Grow-L (500-800µ) caused the inhibitions on protease activities in meagre larvae to range from 16 to 32 DAH. The results point to the inadequacy of commercial diets such as Orange Grow-S, Orange Grow-L and suitability of Orange Start-S, Orange Start-L, Orange Nurse-XS for feeding meagre larvae during the weaning stage. For the mass production of quality juveniles, future studies should take into account the inhibitory effects of commercial diets and feed ingredients before the manufacturing process. A similar approach may be used to determine the most suitable commercial diets for use during the weaning stages of marine fish larvae to obtain the best growth performance and survival.

Keywords: Meagre, *Argyrosomus regius*, Protease activities, Protease inhibitions, Commercial diets, *in vitro*

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Introduction

The sciaenid meagre *Argyrosomus regius* (Asso, 1801) is found in the Mediterranean and Black Sea and along the Atlantic coasts of Europe and the west coast of Africa (Whitehead *et al.*, 1986). Quèmèner (2002) reported that the most important advantage of meagre as a potential candidate for aquaculture was its rapid growth.

Weaning stage which has been known as the transition from live food to micro-diets in aquaculture has a critical importance in the feeding of marine fish larvae. In this stage, aquaculture production depends on fish meal and fish oil known as the main feed ingredients of fish feeds. A shortage was observed in fish meal production while an increase demand has caused increases in fish meal prices. For sustainable aquaculture, there is an urgent need to reduce this dependence with alternative feed ingredients. For this reason, attention has been focused on the replacement of fish meal with more sustainable feed ingredients (Alexis and Nengas, 2001). In this concept, soybean meal is the most important feed ingredient in fish feeds. The main obstacle to the use of high amounts of plant protein sources in fish feeds are low protein quality due to the amino acid imbalances and the presence of antinutritional factors reducing the activity of fish digestive enzymes (Huisman and Tolman, 1992; Krogdahl *et al.*, 2003).

On the other hand, critical feeding stages of marine fish larvae require live food. Munilla-Moran *et al.* (1990) showed that fish larvae had insufficient digestive enzyme capacity for the digestion of exogenous food. Cahu and Zambonino Infante (1994) revealed that survival and growth performance of marine fish larvae fed only on a microdiet were very poor. However, Kolkovski *et al.* (1997) showed that growth and survival in marine larvae showed a marked improvement when live foods were submitted together with microdiets. They investigated the causes of the better utilization of live food by fish larvae when compared with microdiets. Therefore, recent studies have focused on the contribution of digestive enzymes from *Artemia* nauplii and rotifer used as the major food source in critical feeding stages of marine fish larvae (Kurokawa *et al.*, 1998; Garcia-Ortega *et al.*, 2000). Garcia-Ortega *et al.* (2000) revealed that the contribution of digestive enzymes from *Artemia* to the total digestion of food by catfish larvae was less than 1% of total amount of the proteolytic activity measured in the larval gut. Naz (2008) showed that *Artemia* metanauplii provided the highest enzyme contribution from live food to fish larvae. After the above studies, researches were focused on selection of the most suitable feed ingredients and manufacturing technology (Alarcon *et al.*, 1999; Yúfera *et al.*, 2005). Studies indicate a different sensitivity of fish proteases to inhibitors present in feeds, suggesting the need for a preliminary evaluation of the potential useable ingredients in fish

feeds (Moyano *et al.,* 1998; Alarcon *et al.,* 1999).

Until now, the effects of some ingredients commonly used in the production of microdiets on protease activities of seabream larvae and shrimps were studied by some authors (Alarcon *et al.,* 1997; Alarcon *et al.,* 1999). Some authors hypothesized that the limited utilization of microdiets may be related to partial inhibition of protease activities by some dietary factors. Alarcon *et al.* (1999) indicated that ovalbumin significantly reduced (60%) the activity of proteases in 8-day old seabream larvae. Similar results were found when commercially produced microcapsules containing ovalbumin were tested using shrimp proteases (Alarcon *et al.,* 1997).

To increase the success through weaning period, the inhibitory effects of commercial diets must be investigated. Studies on determining inhibitory effects of potential useable feed ingredients and commercial diets on protease activities are important to optimize the larval development stages of *A. regius* and to meet the expectations of hatcheries. Studies have been focused on quality changes of body filet, the ontogeny of digestive system of meagre, the effects of different levels of plant proteins on juvenile meagre and the effects of anaesthesia on juvenile meagre (Poli *et al.,* 2003; Estévez *et al.,* 2011; Serezli *et al.,* 2012; Papadakis *et al.,* 2013). There is only one published study about digestive enzymes of meagre *A. regius* larvae (Süzer *et al.,* 2013). Currently, no study is available on the inhibitory effects of commercial diets on protease activities of meagre *A. regius* larvae. The aim of this research was to gather preliminary data about the potential inhibitory effects of commercial diets on protease activities of *A. regius* larvae using *in vitro* techniques.

Materials and methods

Fertilized eggs of *A. regius* were collected from the broodstock tanks and incubated in conical fiberglass tanks at a temperature of 23.6±0.5 °C. Newly hatched larvae were transferred from the incubators to 7 m^3 fiberglass tanks with black walls until 15 days after hatching (DAH). From 15 to 32 DAH, larvae were stocked in concrete raceway tanks (27 m^3) (stocking density; 75-80 larvae L^{-1} at 0-15 DAH and 10-12 larvae L^{-1} at 16-32 DAH). The rearing tanks were supplied with running sea water that had been filtered through UV filters. Temperature, salinity, oxygen levels and pH were 20.8-24.1°C, 27-40 ppt, 8.4-14.4 and 7.46-7.90, respectively. Rearing tanks were exposed to 18 light (L)-6 dark (D) photoperiod.

Sanolife GWS (INVE Aquaculture nv Hoogveld 91 9200 Dendermonde Belgium) or ω3 Algae® (BERNQAUA nv Hagelberg 3 B-2250 Olen Belgium) were used for green water technique from 3 to 15 DAH.

Rotifer *Brachionus plicatilis* (Muller) were cultured with Algamac Protein Plus (AQUAFAUNE Bio-

Marine Inc. Hawthorne USA) and Sparkle (INVE). The average water temperature and salinity during the culture were 25°C and 25 ppt, respectively. Rotifers were enriched with Spresso (INVE) prior to transferring to the larval feeding tanks. The average water temperature and salinity during the enrichment were 26°C and 28 ppt, respectively.

Artemia nauplii (AF 480; INVE) were cultured at 29°C and 28 ppt. *Artemia* metanauplii (*Artemia* EG; Great Salt Lake Brine Shrimp Cooperative Inc. Utah/USA) were cultured at 29°C and 28ppt. *Artemia* metanauplii were enriched with different enrichments Algamac 3050 (AQUAFAUNE); Red Papper (BERNAQUA) and Spresso (INVE) for 24 hours at 26°C and 28 ppt.

The feeding regime consisted of *B. plicatilis* from 3 to 9 DAH, reaching a maximum concentration of 10-15 prey mL^{-1}, *Artemia* nauplii from 6 DAH onwards, with a maximum density of 2-4 prey mL^{-1}, and *Artemia* metanauplii from 10 DAH onwards, with a maximum density of 1,5-6 prey mL^{-1}. The different sizes Orange Start-S (100-200µ), Orange Start-L (200-300µ), Orange Nurse-XS (300-500µ), Orange Grow-S (300-500µ), Orange Grow-L (500-800µ) of microdiets (INVE) from 16 to 32 DAH were used in the commercial feeding procedure of *A. regius* larvae. Proximate compositions of commercial diets used in the present study are given in Table 1.

Preperation of larval extracts

A. regius larvae fed on commercial feeding procedure were sampled thirteen times, during sampling period (from 3 to 32 DAH). Larvae were taken before the morning feeding and immediately stored in liquid nitrogen (-196°C) to prevent protein autolysis. Larvae sampled according to the above procedure were rinsed in distilled water after thawing and then extracts of larvae were prepared by homogenization of the whole larvae followed by centrifugation (16000g, 30 min., 4°C).

Table 1: Proximate compositions of commercial diets used in present study (INVE).

Composition	Orange Start-S/ Start-L	Orange Grow-S/ Grow-L	Orange Nurse-XS
Crude protein (%)	56	55	55
Crude oils and fats (%)	13	13	13
Crude ash (%)	10	10	13.5
Ash insoluble in hydrochloric acid (%)	2.4	2.4	3
Crude fibre (%)	1	1	1
Σω3 HUFA (mg/g dwt)	40	35	30
DHA/EPA ratio	2	2	2

Extracts of commercial diets

Five commercial diets [Orange Start-S (100-200µ), Orange Start-L (200-300µ), Orange Nurse-XS (300-500µ), Orange Grow-S (300-500µ), Orange Grow-L (500-800µ)] were tested with *in vitro* techniques in the present study. Extracts of commercial diets prepared by homogenization (100 mg mL^{-1} in distilled water) followed by centrifugation (15000g, 10 min.) were used in protease inhibition analyses.

Determination of protease activities of larvae

Total protease activities of *A. regius* larvae were measured as described by Walter (1984) using casein (10 mg mL^{-1}) in 50 mM Tris-HCl buffer at pH 8.5 as the substrate. The mixtures including extracts of larvae and substrate were incubated and then the reaction was stopped by addition of 500µl trichloroaceticacid (TCA) (120 g L^{-1}). One unit of enzyme activity was defined as 1 µg of tyrosine release per minute. All measurements were carried out in triplicate. The soluble protein concentrations of larvae were determined according to Bradford (1976).

Effects of commercial diets on protease activities of larvae

The inhibitory effects of commercial diets on protease activities of *A. regius* larvae were determined by measuring the reduction in protease activity of extracts using a modification of the method described by Garcia-Carreno (1996). The method is based on the measurement of residual protease activity remaining after pre-incubation with different commercial diets such as Orange Start-S (100-200µ), Orange Start-L (200-300µ), Orange Nurse-XS (300-500µ), Orange Grow-S (300-500µ) and Orange Grow-L (500-800µ).

Statistical methods

Results are given as mean±standard error (SE). Comparisons were made using a one-way analysis of variance (ANOVA) test, and differences were considered to be significant at the $p<0.05$ level. The Duncan test for the differences between averages was performed (Bhujel, 2008). SPSS statistical software was used for statistical analyses.

Results

Table 2 shows the changes observed in protease activities of *A. regius* larvae from 3 to 32 DAH. The differences observed in protease activities were statistically significant ($p<0.05$). The lowest and highest protease activities of meagre larvae were 5.95±0.6 U/mg protein and 211.21±12.56 U/mg protein, respectively. Protease activities of *A. regius* larvae tended to decrease from 3 to 5 DAH. After 5 DAH, a sharp increase until 7 DAH and a sharp decrease from 7 to 10 DAH was observed ($p<0.05$). The fluctuations observed in protease activities of meagre larvae were high until 10 DAH.

Table 2: The changes observed in protease activities of meagre larvae (U/mg protein).

Larval age (days after hatching)	Protease activities* (U/mg protein)
3	106.43±9.74^{b}
5	47.93±1.55^{c}
7	211.21±12.56^{a}
10	16.41±2.00^{def}
12	19.68±0.32^{def}
15	5.95±0.60^{f}
17	24.02±1.52^{de}
20	10.49±0.63^{ef}
22	10.59±0.24^{ef}
25	11.23±0.10^{ef}
27	24.53±1.60^{de}
30	12.09±0.52^{ef}
32	28.12±0.49^{d}

*Results are expressed as mean±SE. Different superscripts show significant differences between means of protease activities.

The fluctuations observed in protease activities of *A. regius* larvae were high until 10 DAH and were observed to be lower up to 32 DAH (p<0.05).

Inhibitory effects of Orange Start-S, Orange Start-L, Orange Nurse-XS, Orange Grow-S and Orange Grow-L on protease activities of meagre larvae are given in Fig. 1. The inhibitory effects of commercial diets on protease activities of meagre larvae on 3 DAH was low except for Orange Nurse-XS. From 3 to 5 DAH, a sharp increase was observed in the inhibitory effects of commercial diets. Orange Start-S, Orange Start-L and Orange Nurse-XS had lower inhibition than those of Orange Grow-S and Orange Grow-L at 7 DAH. A sharp decrease in the inhibitory effects of Orange Grow-S and Orange Grow-L on protease activities of meagre larvae was observed while inhibitions of Orange Start-S, Orange Start-L and Orange Nurse-XS tended to increase from 7 to 10 DAH. The inhibitory effects of Orange Start-S, Orange Start-L and Orange Nurse-XS tended to decrease from 10 to 12 DAH. However, Orange Grow-S and Orange Grow-L had higher inhibitions than that of 10 DAH. From 12 to 15 DAH, the inhibitions of commercial diets tended to decrease except for Orange Start-L. A sharp increase in the inhibitions of commercial diets on protease activities were observed on 17 DAH. All commercial diets tested in the present study had lower inhibitions than 30% at 20 DAH (Figs. 1 and 2). After 20 DAH, inhibitions on protease activities of meagre larvae increased up to 22 DAH. The increase observed in Orange Start-S after 20 DAH continued until 25 DAH, however the inhibitions of other diets used in present study tended to decrease from 22 to 25 DAH.

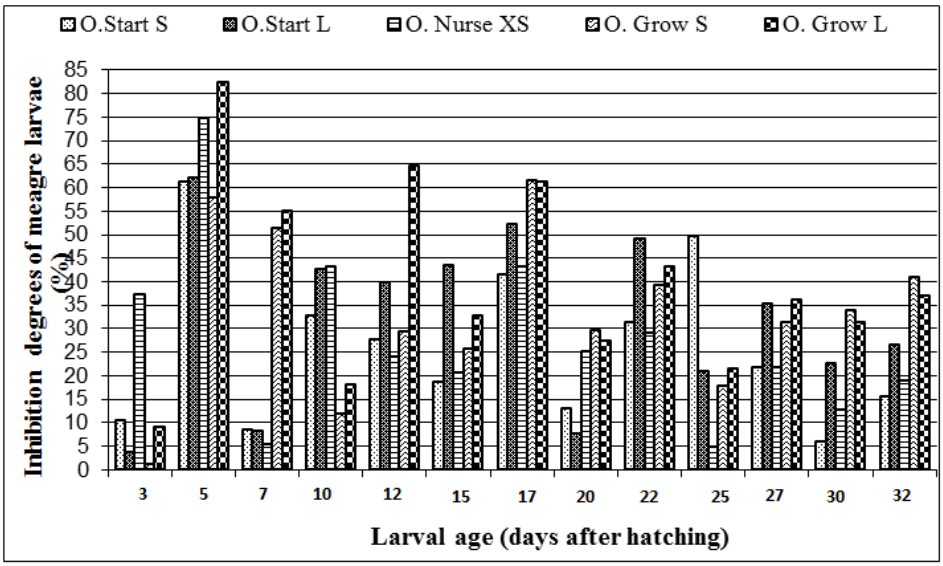

Figure 1: The inhibitory effects of commercial diets (Orange Start-S, Orange Start-L, Orange Nurse- XS, Orange Grow-S and Orange Grow-L) on protease activities of meagre larvae(%).

The inhibition of Orange Start-S up to 27 DAH was decreased while the inhibitions of other diets tended to increase from 25 to 27 DAH. Orange Grow-S and Orange Grow-L remained relatively constant between 27 and 30 DAH. However, the inhibitions of Orange Start-S, Orange Start-L and Orange Nurse-XS were decreased from 27 to 30 DAH. Also, Orange Start-S, Orange Start-L, and Orange Nurse-XS had lower inhibitions at 30 DAH than those of Orange Grow-S and Orange Grow-L. The inhibitions of commercial diets tested on protease activities of meagre larvae tended to increase from 30 to 32 DAH.

Fig. 2 indicates inhibitions lower than 30% of commercial diets on protease activities of meagre larvae. Orange Start-S and Orange Start-L had low inhibitions except for 5, 10, 17, 22 and 25 DAH and 5, 10, 12, 15, 17, 22, and 27 DAH, respectively. Orange Nurse-XS showed lower than 30% inhibitions on protease activities of meagre larvae except for 3, 5, 10, and 17 DAH. Orange Grow-S and Orange Grow-L had high inhibitions except for 3,10,12,15, 20, 25 DAH and 3, 10, 20, 25 DAH, respectively. According to the results of lower than 30% inhibition, Orange Nurse-XS had good performance, especially between 20 and 32 DAH. After Orange Nurse-XS, Orange Start-S and Orange Start-L exhibited good potential.

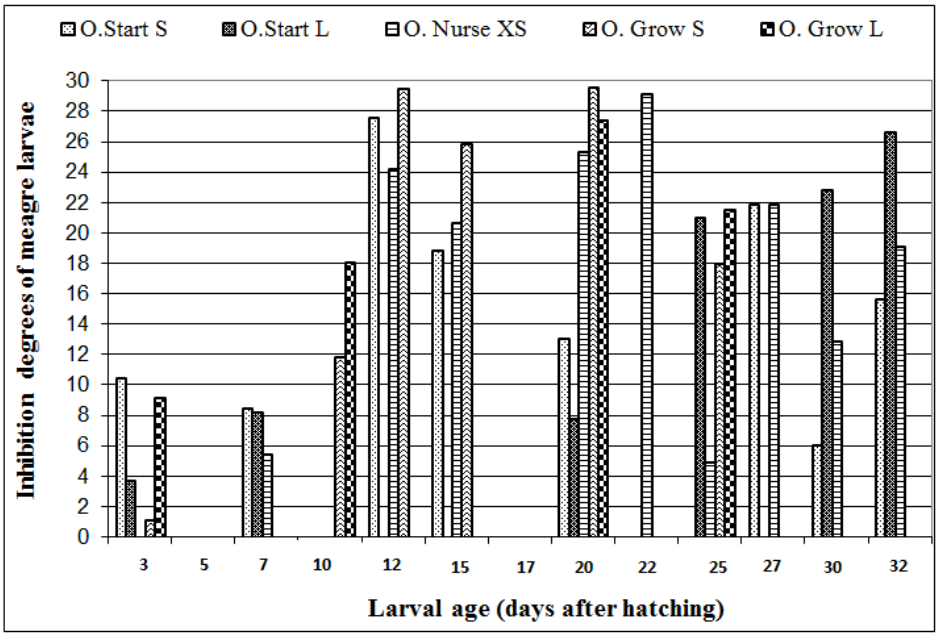

Figure 2: The inhibitory effects of commercial diets such as Orange Start-S, Orange Start-L, Orange Nurse-XS, Orange Grow-S and Orange Grow-L on protease activities of meagre larvae (<30%).

Discussion

The present study provides the first published data about the inhibitory effects of commercial diets on protease activities of meagre larvae. This knowledge is essential when using commercial diets to support the best growth performance and survival as well as the lowest protease inhibitions during weaning stages of meagre larvae.

Currently, there is only one published study about digestive enzymes of meagre *A. regius* larvae (Süzer *et al.*, 2013) and there are none about protease activities and the inhibition effects of commercial diets on protease activities of meagre larvae. The fluctuations in protease activities of *A. regius* larvae were observed from 3 to 32 DAH.

Zambonino Infante and Cahu (2001) indicated that the fluctuations observed in specific activities of marine fish larvae (sea bass, sole and red drum) enzymes is not due to a diminution in enzyme synthesis but is the result of an increase in tissue proteins.

Our results revealed that commercial diets such as Orange Start-S (100-200µ), Orange Start-L (200-300µ), Orange Nurse-XS (300-500µ), Orange Grow-S (300-500µ) and Orange Grow-L (500-800µ) caused the inhibitions on protease activities of meagre larvae from 16 to 32 DAH. It has been hypothesized based on the above findings that the inhibitions observed in protease activities of meagre larvae during weaning may be related to the inhibitor factors found in commercial diets. Alarcon *et al.* (1999) indicated

that ovalbumin significantly reduced (60%) the activity of proteases in 8-day old seabream larvae. Similar results were found when commercially produced microcapsules containing ovalbumin were tested using shrimp proteases (Alarcon *et al.* 1997). Results obtained confirm the existence of protease inhibitors in commercial diets tested in the present work.

The evaluation of commercial diets is crucial to nutrition research and better growth performance for aquaculture species. The results of the present study indicate the differences among the inhibitory effects of commercial diets on protease activities of meagre larvae between 16 and 32 DAH. It is known that fish feed is made from feed ingredients to meet the nutrient requirements of the fish. Deguara *et al.* (2003) mentioned that the knowledge of how different feed ingredients may affect enzyme activity is important and this would provide information on how the choice of ingredients used in manufacturing commercial diets could allow better performance of digestive enzymes.

Differences observed between inhibitory effects of commercial diets tested may be related to the variation in feed ingredients used during the manufacturing of commercial diets. Commercial diet inhibitions may be attributed to the differences in ingredient origin and quality used in formulation. Ingredients used in aquaculture feeds can generally be classified into those being derived from either plant origin or terrestrial animal origin. Krogdahl *et al.* (2003) indicate that the source of the ingredient may be important due to significant problems related to anti-nutritional factors identified in plant origin feed ingredients.

Indeed, meagre larvae exhibit good performance during the larval period when fed with live food. However, dry microdiets contain proteins that are difficult for larvae to digest compared with live foods. The weaning from live food to microdiets induces poor growth due to the high inhibitions observed in protease activities of marine fish larvae. In this concept, the results of the study provide important contributions to determine the most suitable commercial diet for use during the weaning stage of meagre larvae. According to the <30% inhibition results observed in protease activities of meagre larvae, Orange Nurse-XS had better performance than those of other commercial diets, especially between 20 and 32 DAH. After Orange Nurse-XS, Orange Start-S and Orange Start-L exhibited good performance. However, Orange Grow-S and Orange Grow-L had low performance due to the high inhibitions, especially between 27 and 32 DAH. Based on the above findings, Orange Nurse-XS, Orange Start-S and Orange Start-L were recommended for feeding during the weaning stage of meagre larvae but not Orange Grow-S and Orange Grow-L.

In conclusion, three main ideas are presented in this study. First, the results

would point to the inadequacy of commercial diet such as Orange Grow-S (300-500µ), Orange Grow-L (500-800µ) and suitability of Orange Start-S (100-200µ), Orange Start-L (200-300µ) and Orange Nurse-XS (300-500µ) for feeding during the weaning stage of meagre larvae. Second, due to mass production of quality juveniles, the inhibitory effects of commercial diets and feed ingredients during the weaning stages of larvae should be taken into account in future studies before the manufacturing process. Third, a similar approach may be used to determine the most suitable commercial diets for weaning stages of marine fish larvae to obtain the best growth performance and survival. When such data become available, they will serve to determine the most suitable commercial diets for weaning stages of marine fish larvae. For this reason, the inhibitory effects of commercial diets on protease activities of marine fish larvae should be investigated for sustainable aquaculture in future studies.

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