International Journal of Biology; Vol. 6, No. 4; 2014
ISSN 1916-9671 E-ISSN 1916-968X
Published by Canadian Center of Science and Education

Changes of Digestive Enzymatic Activity on Yellowtail Snapper (*Ocyurus chrysurus*) During Initial Ontogeny

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Received: July 18, 2014 Accepted: August 25, 2014 Online Published: August 27, 2014

doi:10.5539/ijb.v6n4p110 URL: http://dx.doi.org/10.5539/ijb.v6n4p110

Abstract

Yellowtail snapper (*Ocyurus chrysurus*) is one of the most important Lutjanids species in the Caribbean due to its great importance in recreational and commercial fishing activities. Studies related to digestive physiology are essential to improve survival after hatching as high mortalities have been reported during larviculture. The aim of this study was to determine the changes in the activities of alkaline and acid proteases, trypsin, chymotrypsin, leucine aminopeptidase, lipase, amylase and alkaline phosphatase in *O. chrysurus* during their initial ontogeny through biochemical techniques. Larvae were fed with live prey (*Nannochloropsis* sp and *Brachionus rotundiformis*) from the opening mouth until 10 days after hatching (DAH), subsequently fed with rotifers and *Artemia sp*. nauplii until 15 DAH and finally were feed with enriched lipid emulsion *Artemia sp*. metanauplii to 42 DAH. Activities of most alkaline digestive enzymes started 5 DAH expressing differentially according to larval growth and larvae live diet. Furthermore, the activity of pepsin in *O. chrysurus* peaked at 34 DAH, suggesting the presence of a functional stomach. It is concluded that the early activity of most alkaline enzymes reflects the contribution of pancreatic and cytosolic enzymes, and that the weaning period could start after 34 DAH after the observed peak of levels.

Keywords: digestive, aquaculture, *Ocyurus chrysurus*, pepsin, ontogeny, alkaline proteases

1. Introduction

Yellowtail snapper (*Ocyurus chrysurus*) belongs to the Lutjanidae family and has significant importance in the recreational and commercial fishing (Faulk, Holt, & Davis, 2005, García-Torcuato, Cervantes-Trujano, & Ancona-Ordaz, 2006; Nirchio et al., 2009). According to Collins (1984), and Faulk et al. (2005), *Ocyurus chrysurus* has been one of the most common and notable species associated to reefs in tropical and subtropical regions, its distribution extends from North Carolina, USA to southeastern Brazil in the western Atlantic Ocean, including the Gulf of Mexico and the Caribbean. Despite their importance a few studies has been carried out focused mainly on phylogenetic relationships and taxonomic revisions of some genus and species. The bottleneck in the commercial culture of this species is a high mortality in the early life stages therefore nutrition studies in larvae and juveniles are vital for a commercial scale production (Moyano, Díaz, Alarcón, & Sarasquete, 1996).

In this sense juveniles production requires constant culture of live feed like microalgae, rotifers and *Artemia*, which are not always the most appropriate as they lack some essential nutrients (Alvarez-González et al., 2006; Alvarez-González et al., 2008; Xiu, Wei, Liang, Zhi, & Shuo, 2009; Jiménez-Martínez et al., 2012). Inert diets on the other hand, have offered easy availability, lower production costs and a viable manipulation, so achieving early weaning in larvae can help reduce production costs of living feed and improve production (Galaviz, García-Gasca,

Drawbridge, Alvarez-González, & López, 2011; Jiménez-Martínez et al., 2012) considering that the feed efficiency depends on fish physiological capabilities for transforming nutrient intake (Alvarez-González et al., 2006; Xiu et al., 2009; Babaei, Kenari, Nazarí, & Gisbert. 2011). This capability is not available at hatching in fishes with indirect ontogeny due to a period of morpho-physiological changes where digestive enzymes are not fully active affecting digestive capacity, growth and survival of the larvae, aspects inadequate to ensure the juveniles production. Thus, the activity of digestive enzymes from hatching to juvenile period can be used as an indicator of nutritional status in early life stage (Moyano et al., 1996; Álvarez-González et al., 2006; Galaviz et al., 2011; Gisbert, Fernández, & Álvarez-González, 2011; Babaei et al., 2011). Several ontogeny studies on digestive physiology during larviculture have been reported as Moyano et al. (1996) in *Sparus aurata*; Zambonino-Infante and Cahu (2001) in *Dicentrarchus labrax*; Alvarez-González et al. (2006) in *Paralichthys californicus*, Alvarez-González et al. (2008) in *Paralabrax maculatofasciatus*; Xiu et al. (2009) in *Miichthys miiuy* and Jiménez-Martínez et al. (2012) in *Centropomus undecimalis*; whose concluded that larvae before the first exogenous feeding may already be able to absorb the nutrients from the diet as well as changes in the enzymatic activity of various digestive enzymes is very similar to other strictly carnivorous marine fishes. Additional studies have been conducted on the early development of the *Menidia menidia* and *Odontestes bonarensis* digestive system to determine the time of maximum enzyme activity (Toledo et al., 2011). Therefore, the aim of this study was to determine the development of various digestive enzymes activities in *O. chrysurus* during initial ontogeny using biochemical techniques.

2. Materials and Methods

2.1 Reproduction and Larvae Culture

This study was conducted in collaboration with the Multidisciplinary Unit of Teaching and Research of UNAM in Sisal, Mérida. A batch of yellowtail snapper reproducers (12 individuals) were kept in an open system, consisting of 4 circular plastic tanks with 20 m^3 capacity. Spawning and hatching (24 h) were in the same broodstock tanks. The Eleuthero-embryos were transferred to three cylinder-conical tanks of 400-liter capacity with constant aeration and controlled temperature for larval rearing.

Once the yolk was absorbed, the larvae were fed daily four times per day (8:00, 12:00, 16:00 and 20:00 h) with microalgae (*Nannochloropsis sp*) and a lipid emulsion (SELCO) enriched rotifers (*Brachionus rotundiformis*) (ER) from the time of mouth opening (3 DAH) until 10 DAH starting a co-feed performed by mixing rotifers with *Artemia sp*. nauplii (AN), at 12 DAH exclusively feed with *Artemia sp*. nauplii; from 25 DAH with lipid emulsion (SELCO) enriched *Artemia sp*. metanauplii (EAN) until 42 DAH. A variable number of individuals were collected (about 100 to 600 larvae), using 500 μm net. The samples were taken on days 1, 2, 3, 4, 5, 7, 8, 10, 12, 15, 17, 19, 21, 23, 26, 28, 30, 32, 34, 38 and 42 DAH. Organisms were anesthetized with overdose of MS-222, liquid nitrogen freeze and stored at -20 ºC, until further use.

2.2 Biochemical Analysis

The whole tissue of the embryos and larvae was cold homogenized using Tris-HCl buffer 50 mmol L^{-1}, pH 7.5 (15 mg mL^{-1}); centrifuged (16 000 g) for 15 min at 4 ºC and the supernatant recovered was stored at -20 ºC until biochemical techniques performed. The concentration of soluble protein in the enzyme extracts was measured using the Bradford technique (1976) using standard bovine serum albumin.

Alkaline protease activity was estimated by the method of Walter (1984) with 0.5% casein as substrate Tris-HCl 50 mmol L^{-1} pH 9 buffer. The acid protease (pepsin activity) was measured using the technique of Anson (1938), in which hemoglobin at 0.5% in Glycine-HCl 0.1 mmol L^{-1}, pH 2 buffer, was the substrate. The mixtures were incubated at 37 ºC, while the reaction was stopped by adding 0.5 ml of TCA at 20%, the absorbance of reaction products was measured at 280 nm light spectrophotometer. The unit of enzyme activity was defined as 1 ug of tyrosine released per minute, based on the molar extinction coefficient (0.005). Trypsin activity was measured as described Erlanger, Largman, and Brodrick (1961) at 25 ºC, BAPNA (N-α-benzoyl-DL arginine p-nitroanilide) as substrate in Tris-HCl 50 mmol L^{-1} pH 8.2 buffer (with $CaCl_2$ 10 mmol L^{-1}). Chymotrypsin activity was determined using the method of DelMar, Largman, Brodrick, & Geokas (1979) at 25 ºC with SAAPNA (N-succinyl-ala-ala-pro-phe-p-nitroanilide) as substrate in DMSO as 10 mmol L^{-1} and Tris-HCl 100 mmol L^{-1} (with $CaCl_2$ 10 mmol L^{-1}) at pH 7.8. Carboxypeptidase A activity was measured using the method described by Folk and Schirmer (1963), using as a substrate (Hippuryl-L-phenyl-alanine) in 0.025 M Tris- HCl (with 0.5 mol L^{-1} NaCl) at pH 7.5. Leucine-aminopeptidase activity was examined in Leucine p Nitroanilide in 0.1 mmol L^{-1} dissolved in DMSO, using 50 mmol L^{-1} sodium phosphate buffer, pH 7.2 at 25 ºC (Maraux, Louvard, & Baratti, 1973). The techniques described above stopped reactions with acetic acid at 30%, while the enzyme activity is

defined as 1 ug nitroanilide released per minute using a molar extinction coefficient (8.8) for trypsin, chymotrypsin and leucine-aminopeptidase.

For identifying α-amylase enzymatic activity, 2% starch was used as a substrate in a phosphate-citrate buffer, plus 0.1 M plus NaCl 0.05 mol L^{-1} at a pH 7.5, the absorbance was quantified at 600 nm (Robyt & Whelan, 1968). Lipase was measured in the substrate β-naphthyl caprylate 200 mM (in 50 mmol L^{-1} Tris- HCl pH 7.2 and 100 mmol L^{-1} sodium taurocholate) as described Versaw et al. (1989), the incubation was 30 min, and the reaction was interrupted with TCA 0.72 N, 100 mmol L^{-1} fast blue was added and finally clarified with a solution of ethanol: ethyl acetate (1:1 v/v) mixture, the unit used in this case was specified in accordance with the absorbance readings at 540 nm, as 1 mg of naphthol released per minute with a molar extinction coefficient of 0.02.

The alkaline phosphatase activity was estimated with 4-nitrophenyl phosphate as substrate at pH 10.1 with NaOH-glycine buffer, according to Bergmeyer (1974). The unit of activity was defined as 1μg of nitrophenol released per minute. The specific activity of the extract was expressed using the following equations: 1) Units per ml = (Δabs x final reaction volume (mL)) (MEC x time (min) x extract volume (mL))$^{-1}$; 2) Units per mg of protein = Units per mL x mg of soluble protein^{-1}; 3) Units per larvae = Units per mL x number of larvae per mL^{-1}. Δabs represents the increase in absorbance at a given wave amplitude and MEC represents the molar extinction coefficient for the reaction product (mL μg^{-1} cm^{-1}). All tests were performed in triplicate.

3. Results

The values in the enzymatic activity of the yellowtail snapper *Ocyurus chrysurus* were determined based on the specific and individual statistical differences represented by each enzyme. First of all, the activity of the pepsin enzyme per mg of protein remained inactive in the first 15 DAH, subsequently presented a slight increase at 19 DAH (365.2 mU mg protein^{-1}) remaining constant, reaching its maximum level at 34 DAH (2186.8 mU mg protein^{-1}) until 42 DAH (Figure 1a). Moreover, the activity per larvae units in pepsin, remained with no activity at the first 15 DAH, rising and fluctuating its values between 19 to 32 DAH, observing its maximum peak of activity at 34 DAH (5.5 U larvae^{-1}), finally a slight decline to 42 DAH was shown (Figure 1b). Alkaline protease activity per mg of protein started at 5 DAH (8.1 mU mg protein^{-1}) with an increase at 19 DAH reaching its highest level at 28 DAH with subsequently great fluctuations decreasing at 42 DAH (Figure 1c). Moreover, alkaline protease activity in units per larvae showed a similar pattern starting at 5 DAH, increasing at 19 DAH with maximum activity at 26 DAH (2.4 U larvae^{-1}), decreasing activity up to 42 DAH (1.3 U larvae^{-1}) (Figure 1d). Trypsin activity per mg of protein was active from 4 to 11 DAH, being inactive again at 12 DAH, afterward increasing fluctuations was observed from 15 DAH reaching its maximum activity at 42 DAH (765.9 × 10^{-3} mU mg protein^{-1}) (Figure 1e). Regarding the trypsin activity in units per larvae, it started at 5 DAH with fluctuations reaching the lowest value at 12 DAH, restarting its activity at 15 DAH showing slight fluctuations up to their maximum level at 42 DAH (3953.1 mU larvae^{-1}) (Figure 1f). Concerning chymotrypsin per mg of protein, its activity started at 8 DAH showing a small decrease from 10 to 12 DAH, reaching their maximum activity between 17 - 32 DAH (18.6 - 20.0 mU × 10^{-3} mg protein^{-1}) (Figure 1g). Moreover, chymotrypsin in units per larvae begins its activity from 17 DAH, reaching its peak at 42 DAH (5.4 mU larvae^{-1}) (Figure 1h). For leucine-aminopeptidase mg of protein, it was observed that starting from 7 DAH, it began its activity, which showed a decline between 10 and 12 DAH thus increasing to 15 DAH, and later on, reach a peak level of 34 DAH (72643.8 × 10^{-3} mU mg protein^{-1}) (Figure 1i). Finally, leucine aminopeptidase in units per larvae shows that the first 5 DAH remained inactive, starting at 7 DAH increasing activity considering a decline between 10 to 12 DAH, subsequently they showed slight fluctuations, reaching its maximum level 26 DAH (273.3 mU larvae^{-1}) decreasing the following days and increasing again at 42 DAH (238.0 mU larvae^{-1}) (Figure 1j).

Values of lipase in mg protein showed maximum activity from the first DAH (0.3 mU mg protein^{-1}), so subsequent days their activity decreased with slight fluctuations up to 12 DAH (0.07 mU mg protein^{-1}), and subsequently increased its activity from 15 DAH, decreasing again at 19 DAH (0.04 mU mg protein^{-1}), keeping the same values with slight fluctuations next day (Figure 2a). With regard to lipase in units per larvae, it started its activity from the first DAH, with significant fluctuations to reach its maximum level at 7 DAH (2.0 U larvae^{-1}), suddenly descending down to 8 DAH and increasing to 10 DAH, therefore from 12 DAH, the activity decreased again remaining constant in their values up to 42 DAH (Figure 2b). In terms of the activity of alkaline phosphatase per mg protein, it began at 3 DAH, significantly increasing up to 8 DAH (56.6 mU x 10^{-3} mg protein^{-1}) and declining at 12 DAH, then their values continued fluctuating up to 30 DAH, so at 32 DAH, a maximum activity was shown, to values up to 65.0 × 10^{-3} mU mg protein^{-1}, and finally a few days later, it showed a slight decline down to 42 DAH (Figure 2c). Therefore, alkaline phosphatase in units per larvae was active from 7 DAH, increasing significantly to 10 DAH (10048.0 × 10^{-3} mU larvae^{-1}) and showing a reduction at 12 DAH, then decreased with slight fluctuations to 26 DAH and increased its activity again until reaching its peak at 32 DAH with values of 11434.4 × 10^{-3} mU larvae^{-1} (Figure 2d). Finally, the

α-amylase activity in mg of protein remained low until 12 DAH, increasing to 15 DAH and achieving significant values at 17 DAH (0.02 U mg protein^{-1}), therefore the enzyme activity decreased significantly between 19 to 26 DAH, and increased considering its maximum level at 28 DAH (0.01 U mg protein^{-1}), then it fluctuated slightly and decreased down to 42 DAH (Figure 2e). Regarding amylase in units per larvae, it shows low activity in the first days after hatching, presenting a slight fluctuation at 12 DAH and raising its activity at 15 DAH. Subsequently it shows significant value of 0.03 U larvae^{-1} at 17 DAH, and suddenly dropped to 21 DAH, in the next days its activity rose to 42 DAH showing a maximum level of activity (0.05 U larvae^{-1}) (Figure 2f).

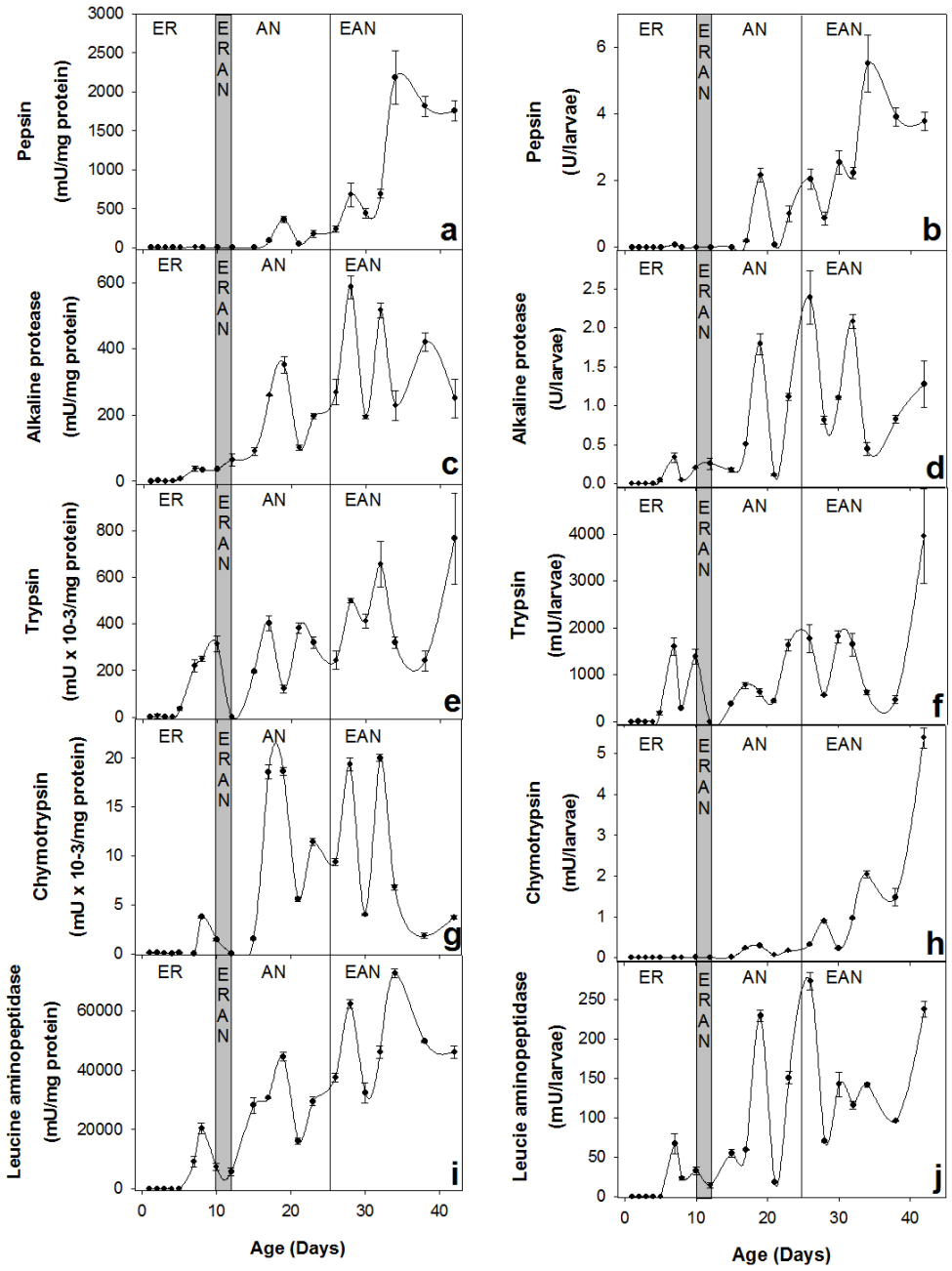

Figure 1. Digestive enzyme activity in yellowtail snapper, *Ocyurus chrysurus* during its ontogeny. a) Pepsin specific activity. b) Pepsin Individual activity. c) Alkaline protease specific activity. d) Alkaline protease individual activity. e) Trypsin specific activity. f) Trypsin individual activity. g) Chymotrypsin specific activity. h) Chymotrypsin individual activity. i) Leucine aminopeptidase specific activity. j) Leucine aminopeptidase individual activity. ER, enriched rotifers; ERAN, enriched rotifers and *Artemia* nauplii; AN, *Artemia* nauplii; EAN, enriched *Artemia* nauplii

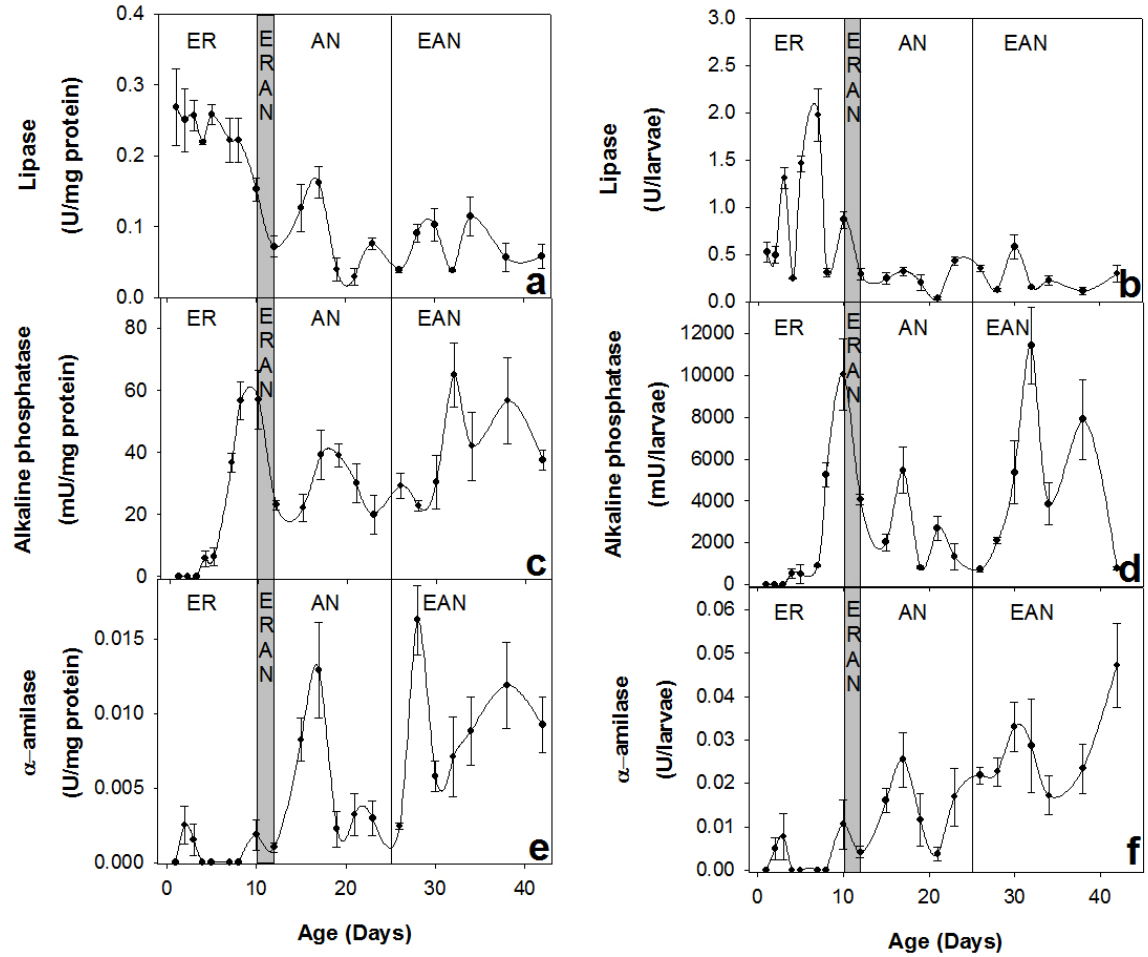

Figure 2. Digestive enzyme activity in yellowtail snapper, *Ocyurus chrysurus* during its ontogeny. a) Lipase specific activity. b) Lipase individual activity. c) Alkaline phosphatase specific activity. d) Alkaline phosphatase individual activity. e) α-amylase specific activity. f) α-amylase individual activity. ER, enriched rotifers; ERAN, enriched rotifers and *Artemia* nauplii; AN, *Artemia* nauplii; EAN, enriched *Artemia* nauplii

4. Discussion

Yellowtail snapper is one of the most important commercial species in the region of Yucatán peninsula. This species has been studied in order to implement its culture at a commercial level, which today is achieved from a pilot scale although many aspects are unknown specially those related to nutrition in the early life stage where the action of digestive enzymes is really important. The results of this study in yellowtail snapper showed that from day five after hatch, most alkaline digestive enzymes started and expressed differentially as the larvae grew and changes were made from live food to composed diet. Previous studies on the digestive capacity of marine fish larvae focused in the relationship among live food diets with anatomical changes during metamorphosis. Artificial diets provide adequate quality in function of digestive enzymes present in larvae, improving survival, growth and resistance of this species (Jiménez-Martínez et al., 2012). Our results are similar to those reported for other species, where the digestive process in fish larvae in alkaline conditions usually begins with the contribution of pancreatic and cytosolic enzymes (Xiu et al., 2009). In this context, the presence of alkaline proteases, trypsin and specific chymotrypsin, detected in the first days following the mouth opening was similar to the results of carnivorous fish such as *P. californicus* (Alvarez-González et al. 2006), *P. maculatofasciatus* (Alvarez-González et al. 2008) and *C. undecimalis* (Jiménez-Martínez et al., 2012), where the earliest activity corresponds to alkaline proteases, as the acid protease activity is closely related to the occurrence of a functional stomach which takes place later (Moyano et al., 1996; Alarcón-López & Martínez-Díaz, 1998; Zambonino Infante & Cahu, 2001; Alvarez-González et al. 2008; Jiménez-Martínez et al., 2012).

Moreover, changes in alkaline proteases activities imply that specific activity of these enzymes are due to the growth and development of organs and new tissues as well as a response to dietary change during larval development (Zambonino-Infante & Cahu 1994; Alarcón-López & Martínez-Díaz, 1998; Martínez. Moyano, Fernández-Díaz, & Yúfera, 1999; Ribeiro, Zambonino-Infante, Cahu, Dinis, 2002; Alvarez-González et al., 2008; Xiu et al., 2009).

Particularly trypsin activity generally started increasing or decreasing in transitions from exogenous feeding or during changes in supplied diet suggesting that these enzyme activities can be modulated by feeding patterns. In the same study, it was shown that the activity of trypsin is less relevant for food digestion during and following weaning, suggesting a greater role for other enzymes at this stage (Moyano et al., 1996; Alvarez-González et al., 2008; Xiu et al., 2009). Also after the onset of exogenous feeding, the activity of leucine aminopeptidase is modulated by the diet, as the above mentioned by trypsin, because it shows a steep rise with slight fluctuations caused by the evolution thereof. Likewise, this increase can be explained by the different nutrient composition of rotifers and *Artemia* integrated into their diet, which, as mentioned above are not the most suitable for the culture of many of the marine fish species (Moyano et al., 1996; Alvarez-González et al., 2006; Alvarez-González et al., 2008; Xiu et al., 2009).

In the present work, the activity of pepsin of *O. chrysurus* peaked at 34 DAH, this is in similar to results from other marine fishes such as *Solea senegalensis* (Martínez et al., 1999), *P. californicus* (Alvarez-González et al., 2006), *P. maculatofasciatus* (Alvarez-González et al., 2008), *Scophthalmus maximus* (Tong et al. 2011) y *C. undecimalis* (Jiménez-Martínez et al., 2012). The absence of a functional and morphologically different stomach during the first days after hatching is common for marine fish larvae. A peak of pepsin specific activity indicates the presence of a fully functional stomach, which coincides with the histological changes in the stomach and a capacity of acid digestion of proteins present in the food (Moyano et al., 1996; Zambonino Infante & Cahu, 2001; Tong et al., 2012; Jiménez-Martínez et al., 2012). At the beginning of their development the larvae have an undifferentiated straight tube, which later develops into a differentiated gastrointestinal tract lined whith gastric cells and at this stage the weaning period may start (Zambonino Infante & Cahu, 2001; Jiménez-Martínez et al., 2012, Moyano et al., 1996).

Ontogenic changes were also reflected in the lipase activity, which was present from the first DAH and latrer flactating and decreasing. These fluctuations may be related to changes in the diet (Jiménez-Martínez et al., 2012). Also, the early activity of lipases in larvae and Eleuthero-embryos of some species of marine fishes has been reported by several authors (Oozeki & Bailey, 1995; Alvarez-González et al., 2008). However, the presence of the enzymatic capacity before the first feeding suggests that these activities were not induced by food (Zambonino-Infante & Cahu, 2001). Therefore, the current changes in the lipase activity showed difference compared to other enzymes since the first day of its presence and probably is related to the existence of non-pancreatic lipase (Oozeki & Bailey, 1995), according with Jiménez-Martínez et al. (2012) there are two types of bile salt-dependent lipase, the first one is active during the embryonic stage, thereby aiding in the digestion of lipids from yolk, while the second type is directly secreted by the pancreas to digest food lipids. The reduction in the specific activity of the enzyme is not due of a reduction in enzyme synthesis perhaps is the result of an increase proteins of the tissue related to the anatomic and physiologic changes in fishes species with metamorphosis (Zambonino-Infante & Cahu, 2001).

Amylase activity before the first ingestion of food was similar to other marine fishes as *Theragra chalcogramma* (Oozeki and Bailey 1995); *Sparus aurata* (Moyano et al., 1996); *Solea senegalensis* (Martínez et al., 1999) and *Miichthys miiuy* (Xiu et al., 2009), followed by an alternative decrease and increase fluctuating activity which indicates an early presence of amylase activity triggered by specific stimulation discarding mechanism of diet, as may correspond to differences in nutritional requirements and the presence of glycogen in the yolk itself, prior to absorption (Zambonino-Infante & Cahu, 1994; Cahu & Zambonino-Infante 1995; Moyano et al., 1996; Zambonino-Infante & Cahu, 2001; Ribeiro et al., 2002; Xiu et al., 2009). Therefore, the amylase activity may be closely related to its ability to digest carbohydrates present in microalgae as food for zooplankton, and other factors, such as nutrients or digestibility (Moyano et al., 1996). Likewise, it is attributed that the amylase activity may be determined not only by the morphological changes in the digestive system, as a consequence of the development of the larvae, but also by other key factors such as eating habits, live food exogenous enzymes, or even temperature during culture (Oozeki & Bailey, 1995; Zambonino-Infante & Cahu, 2001; Xiu et al., 2009). Although its specific function has not been fully understood in the early development stages of fish, it has been considered as an indicator of the maturation of the digestive system when their activity decreases abruptly, indicating that the carbohydrate requirement is not necessary, since metabolic energy is obtained mainly from proteins (Jiménez-Martínez et al., 2012).

Finally, phosphatases are an important detoxification system involved in mineralization and nutrition processes, also in phosphate transport and hydrolysis of the phosphorylated proteins (Moyano et al., 1996). Alkaline phosphatase is mainly distributed in the striated edge of epithelial cells found in the foregut and can be associated with nutritional absorption in the intestine (Alvarez-González et al., 2006; Xiu et al., 2009). This enzyme initiates from the four DAH, which indicates a high activity in the larvae during the yolk sac stage, demonstrating that the intestines have already reached a high absorption capacity in this period, especially when leucine aminopeptidase enzyme increases. So it is interpreted as an absorption capacity index of fish larvae at this stage. As the larva develops, slight fluctuations were shown tending to increase the enzyme activity showing a pattern of continuous increase development or a succession of increases and decreases with age, which could be interpreted in response to change in diet during larval development (Alvarez-González et al., 2006; Xiu et al., 2009).

5. Conclusion

In conclusion, this study demonstrated an early enzymatic activity for alkaline proteases, lipases, amylases and alkaline phosphatases, with slight fluctuations presented throughout its development and changes in established diet, suggesting early maturation of the digestive system, and favorable response to the diet supplied. Further monitoring of the formulation of an applied compound diet is required to provide more information for larvae culture. Moreover, the delayed response of enzyme activity by pepsin, demonstrates the formation of a functional stomach coinciding with histological changes in the stomach and the top of the acid digestion, *Ocyurus chrysurus* presents a complete maturation of the digestive system from 34 DAH suggesting an adaptation to the digestion of complete protein diets which would include a reduction in the preference for live food diets with high costs.

Acknowledgments

We thank Claudia Durruty Lagunes and Jaime Suárez Bautista for their technical assistance. To the Programa Integral de Fortalecimiento Institucional for the financial support, which are public resources and it is forbidden their use for political or personal porpoises.

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