University of Algarve
Faculty of Marine and Environmental Sciences

The influence of inert diets on digestive capacity of fish larvae species: *Diplodus sargus* and *Solea senegalensis*

Inês Maria dos Santos Guerreiro

Masters in Aquaculture and Fisheries
Specialization Aquaculture

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Thesis supervision by:

Internal coordinator: Doutora Laura Ribeiro
(Centro de Ciências do Mar - CCMAR)
Professora Doutora Maria Teresa Dinis
(Universidade do Algarve)

External coordinator: Dr. Pedro Marques Pousão Ferreira
(Investigador auxiliar e responsável pela Estação Piloto de Piscicultura de Olhão do Instituto Nacional dos Recursos Biológicos – IPIMAR)

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Abstract

The present study aimed to analyse the effect of weaning on white seabream and Senegalese sole digestive capacity by analysing pancreatic and intestinal digestive enzymes. For white seabream an earlier introduction of the diet was analysed, so diet was introduced at 20 and 27 days after hatching (DAH), respectively, W20 and W27 treatments. For sole (30 DAH) two weaning strategies were studied, namely sudden weaning, SW treatment, and 5 days co-feeding, CF treatment.

The experiment with white seabream was divided in two periods, until 20 days where the pattern of digestive enzymes activity was analysed; after 20 DAH where the introduction of inert diet was analysed for 3 weeks period, ending at 41 and 48 days respectively for W20 and W27 treatment. For sole, the experimental period was carried out between 30 and 60 DAH.

Trypsin, amylase, lipase, pepsin, alkaline phosphatase, acid phosphatase, aminopeptidase and leucine-alanine peptidase, were analysed on different larval segments (whole larvae, abdominal cavity, digestive tract segment and brush border).

Until 20 DAH trypsin, alkaline phosphatase, leucine-alanine, aminopeptidase and acid phosphatase specific activities increased significantly, while pepsin, amylase and lipase kept relatively constant until 20 DAH.

Regarding white seabream weaning, W27 treatment exhibited higher growth than W20 treatment. Nonetheless, no significant differences were observed on fish larvae digestive enzymatic capacity between treatments 3 weeks after inert diet introduction, and although larvae from W20 treatment were more affected initially they were able to recover to similar levels of activity. This study suggested that inert diet can be included in the feeding regime of white seabream as early as 20 DAH

Sole post larvae from SW treatment exhibited higher growth and survival rate than CF treatment. Trypsin, alkaline phosphatase and leucine-alanine specific activities decreased until 40 DAH, while amylase, pepsin and aminopeptidase kept more or less constant until 40 DAH, where no differences were observed between treatments. The total activity tended to decrease until 40 DAH, but without significant differences between treatments. At 60 DAH there were not significant differences in enzymes specific and total activities between treatments.

The decrease observed for digestive enzymes total activity reflected the poor condition of sole larvae from both treatments. Both strategies of weaning resulted in poor results.

Key words: *Diplodus sargus, Solea senegalensis,* digestive enzymes, weaning, ontogeny, fish larvae, digestive capacity.

Resumo

O conhecimento da fisiologia digestiva de larvas de peixes marinhos ao longo do seu desenvolvimento é crucial para compreender as capacidades digestivas das espécies, e assim permitir a sua produção em aquacultura.

O sargo, *Diplodus sargus*, e o linguado, *Solea senegalensis*, são duas espécies de elevado valor económico e com potencial de cultivo, sendo por isso importante estudar aspectos relacionados com a sua fisiologia digestiva. Com este trabalho pretendeu-se estudar a influência do alimento inerte na capacidade digestiva do sargo e do linguado, através da quantificação da actividade de diferentes enzimas digestivos. No caso do sargo, analisou-se o efeito de uma adaptação precoce ao alimento inerte, ao introduzir-se a dieta aos 20 dias após a eclosão (DAE; W20) e comparando com o tratamento em que a dieta foi introduzida aos 27 DAE (W27; normal/controlo). No caso do linguado avaliou-se a estratégia de adaptação ao alimento inerte, nomeadamente uma adaptação súbita – tratamento SW – ou uma co-alimentação durante 5 dias – tratamento CF –, ambos com início aos 30 DAH.

No caso do sargo consideram-se duas fases experimentais. Na primeira fase que decorreu até aos 20 dias, analisou-se o padrão da actividade enzimática ao longo do desenvolvimento das larvas. Na segunda fase, avaliou-se o efeito de uma adaptação precoce ao alimento inerte nos enzimas digestivos durante 3 semanas, terminando o ensaio aos 41 e 48 DAE, respectivamente, para o tratamento W20 e W27. O plano alimentar consistiu em rotíferos (4-26 DAE), *Artemia* naupli (13-19 DAE), *Artemia* metanaupli (16-26 DAE para o tratamento W20 e 16-33 DAE para o tratamento W27) e alimento inerte (20-41 DAE para o tratamento W20 e 27-48 para o tratamento W27).

Em relação ao linguado, o período experimental decorreu entre os 30 e 60 DAE consistindo o plano alimentar em *Artemia* (30-34 DAE para o tratamento CF) e alimento inerte (30-60 DAE).

No caso do sargo, realizaram-se amostragens aos 0, 2, 9, 13 e 20 DAE para caracterizar o padrão de actividade digestiva. Enquanto no ensaio de adaptação ao alimento inerte, realizaram-se amostragens no início da adaptação, uma e três semanas após a introdução do alimento inerte. Em relação ao linguado, efectuaram-se amostragens aos 30, 35, 40, 50 e 60 DAE pós-larvas de linguado.

No sargo analisou-se a tripsina, a amilase, a pepsina, a lipase, a fosfatase alcalina, a leucina-alanina peptidase, a aminopeptidase e a fosfatase ácida. No linguado apenas foi analisado a tripsina, amilase, pepsina, fosfatase alcalina, aminopeptidase e leucina-alanina peptidase. Os dados da actividade enzimática foram apresentados como actividade específica: U mg protein^{-1} e actividade total: U larva^{-1}.

A actividade enzimática foi determinada em diferentes segmentos, ao longo do desenvolvimento larvar. No sargo, até aos 20 DAE utilizou-se todo o corpo da larva para a análise enzimática, enquanto as larvas mais velhas foram dissecadas a fim de se obter a porção abdominal. No linguado, até aos 40 DAE as pós-larvas foram dissecadas obtendo-se a porção abdominal, enquanto nas idades posteriores as pós-larvas foram dissecadas para se obter o segmento pancreático e o intestinal, o qual foi ainda utilizado para obter o prato estriado do intestino.

As larvas de sargo apresentaram um crescimento exponencial tanto em peso como em comprimento. Até aos 20 DAE as actividades específicas da tripsina, fosfatase alcalina, leucina-alanina peptidase, aminopeptidase e fosfatase ácida aumentaram significativamente, enquanto as actividades específicas da pepsina, amilase e lipase mantiveram-se relativamente constantes. As actividades da lipase e leucina-alanina só foram detectadas aos 9 DAE. A actividade da pepsina teve o maior valor de actividades aos 0 DAE mas aos 2 DAE não se detectou actividade. A actividade total dos enzimas estudados aumentou significativamente até aos 20 DAE.

No ensaio de adaptação ao alimento inerte, as larvas de sargo do tratamento W27 comparativamente às larvas do tratamento W20 apresentaram um maior crescimento em peso e comprimento e uma sobrevivência superior. Três semanas após a introdução do alimento inerte no plano alimentar do sargo, as larvas do tratamento W20 apresentavam uma tendência em ter uma actividade enzimática superior, embora sem apresentarem diferenças significativas entre os tratamentos. Neste mesmo período, as larvas do tratamento W20 comparativamente com as larvas do tratamento W27 apresentaram no prato estriado uma maior actividade específica da aminopeptidase. A actividade total dos enzimas estudados aumentou significativamente até ao final da experiência, sendo significativamente maior nas larvas do tratamento W27. No entanto, analisando o aumento relativo da actividade dos enzimas digestivos, observou-se que as larvas do tratamento W20 exibiram um aumento relativo após as três semanas da introdução do alimento inerte no plano alimentar. Três semanas após a introdução do alimento inerte não se observaram diferenças significativas no índice de maturação dos enterócitos para ambos os tratamentos.

Em relação ao linguado, as pós-larvas do tratamento SW comparativamente às pós-larvas do tratamento CF apresentaram um maior crescimento em peso e comprimento e uma sobrevivência superior. As actividades específicas da tripsina, fosfatase alcalina e leucina-alanina peptidase decresceram até aos 40 DAE, enquanto as actividades específicas da amilase, pepsina e aminopeptidase se mantiveram relativamente constantes até os 40 DAE. A esta idade não existiram diferenças

significativas entre as actividades específicas dos tratamentos. As actividades específicas determinadas no prato estriado decresceram até aos 40 DAE, mas sem se observarem diferenças significativas entre os tratamentos. A actividade total até aos 40 DAE teve uma tendência decrescente mas sem diferenças estatísticas entre os tratamentos. Considerando a fosfatase alcalina, aos 40 DAE a percentagem de maturação dos enterócitos é significativamente superior nas larvas do tratamento SW.

Aos 60 DAE não ocorreram diferenças significativas nas actividades específicas dos enzimas entre os tratamentos do linguado. Os valores de actividade total analisados no pâncreas e intestino mantiveram-se quase inalterados entre as duas idades e tratamentos analisados. Aos 60 DAH não ocorreram diferenças significativas entre tratamentos relativamente à percentagem de maturação dos enterócitos.

O padrão de actividade dos enzimas digestivos estudados no sargo esteve relacionado com a organogénese e o tipo de alimento usado nos diferentes estados de desenvolvimento. Este estudo permitiu verificar que antecipando a introdução do alimento inerte no plano alimentar das larvas de sargo, neste caso aos 20 DAE, a capacidade digestiva não é afectada, uma vez que as larvas no final do período experimental apresentavam níveis de actividade enzimática semelhantes aos apresentados pelas larvas do tratamento W27.

A actividade enzimática no linguado não foi afectada pelo tipo de desmame e, quando o foi, no final do período experimental o nível de actividade enzimática era semelhante para ambos os tipos de desmame. O método de desmame mais ambicionado pela indústria é o desmame súbito já que permite uma redução na utilização de alimento vivo. Neste estudo o tipo de desmame que menos afectou fisicamente as pós-larvas foi o desmame súbito, mas os resultados não permitem concluir que este seja o melhor método, já que ambas as estratégias alimentares produziram fracos resultados. O decréscimo observado na actividade enzimática total reflectiu a baixa condição física exibida pelas pós-larvas de ambos os tratamentos.

Palavras-chave: *Diplodus sargus, Solea senegalensis,* enzimas digestivas, desmame, ontogenia, larvas de peixe, capacidade digestiva.

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1. Introduction

To successfully rear aquaculture species through the larval stage is essential to know their nutritional physiology (Zambonino-Infante and Cahu, 2007). Nowadays aquaculture species have assumed a great importance regarding the scarcity of some marine fish resources and the increasing demand of food to support the world population. An adequate nutrition is essential to be successful in rearing fish larvae and post-larvae and to quickly achieve big and healthy adults and this depends on the effective ingestion, digestion and assimilation of the right nutrients contained in the diet (Lazo *et al*., 2007).

Nowadays aquaculture industry searches for fish species with potential to be reared with sustainable methodologies, in other words, fish which is cultivated in a healthier manner. Mesocosm technology combines intensive and extensive aquaculture, being a semi-intensive technique, that is considered a potential sustainable method which solves many problems found in intensive and extensive methods such as biological and technical ones (Papandroulakis *et al*., 2004). Papandroulakis *et al.* (2004) successfully reared *Diplodus sargus sargus* with a mesocosm technology. Solea *senegalensis* and *Diplodus sargus* seem to be species that presents potential for this type of production.

The first period of cultivation of marine species is entirely dependent of live feeds, and this raises the production costs (Rosenlund *et al.,* 1997). In fact one of the main objectives of aquaculture is a complete substitution of live feed by inert diets, although it was only accomplished with European sea bass larvae (Zambonino-Infante and Cahu, 2007). Live feed provides better growth and survival in early stages of fish larvae but has some disadvantages such as they may act as vectors of diseases, the nutritional value can be variable, the nutritional quality is difficult to manipulate and above all they are time-consuming to produce and consequently expensive (Pousão-Ferreira *et al*., 2003; Ribeiro *et al.,* 2005). Substitution of live feed by compound diets is essential to reduce the production costs and for a sustainable production of high and constant quality juveniles (Cahu and Zambonino-Infante, 2001). So, today there are huge efforts to reduce the period of live feed, and introduce compound diet as early as possible.

Among the different technologies used to reduce the period of live feeds, a period of co-feeding is frequently adopted, because beyond reducing the period of live feed the aim is to obtain quality juveniles that are yet dependent of live feed. Co-feeding has been demonstrated to improve larval performance, when compared with feeding only

dry or live feed, and to wean in a shorter time (Roselund *et al.*, 1997; Pousão-Ferreira *et al.*, 2003; Engrola *et al.,* 2007). For Kolkovski *et al.* (1997), co-feeding presents advantages in weaning fish larvae since visual and chemical stimuli increases inert diet ingestion and there are a biochemical influence of live feed in larvae digestion and assimilation. A more suitable nutrient supply is given when co-feeding dry and live feed (Roselund *et al.*, 1997; Cañavate & Fernández-Díaz, 1999). Other advantage of co-feeding is that the presence of live feed together with inert diet makes larvae accept more easily the inert diet when the live feed is withdrawn (Cañavate & Fernández-Díaz, 1999; Pousão-Ferreira *et al.*, 2003). The period of co-feeding changes depending on species, last only a few days in some species or several weeks in others (Conceição *et al.,* 2007).

The study of the ontogeny and activity of enzymes in fish larvae might be of great importance to understand the digestive process and to reduce the period of live feed (Cara *et al.*, 2003; Suzer *et al,* 2007). Different levels of enzymes and enzyme activities could indicate different nutrient requirements by larvae (Ribeiro *et al*, 2008), and this information can help to define adequate nutritional protocols for specific ages (Tengjaroenkul *et al*, 2002; Tramati and Mazzola, 2005). Changes in the diet are not the principal reason for changes in the enzymatic activity during ontogeny, but determine the plateau level of enzymes, being fishes able to modulate their enzymatic activity as the characteristics of feed change (Cahu and Zambonino-Infante, 1994).

During development, digestive enzyme capacity has been used to evaluate the larval digestive capacity, as an indicator of larval development and as a predictor of future survival (Cara *et al.*, 2003). It has been proposed that proteolytic enzymes activities can influence the maximum growth rate that fish can attain (Blier *et al*, 2002).

Digestion involves several enzymes, such as trypsin, aminopeptidase, amylase, alkaline phosphatase, acid phosphatase, pepsin, lipase, leucine-alanine peptidase. These enzymes can be divided in proteolytic enzymes and lipolitic enzymes.

Proteolytic enzymes namely trypsin, aminopeptidase, amylase, alkaline and acid phosphatase, pepsin and leucine-alanine peptidase can be found in three areas of the digestive tract, stomach, pancreas and intestine.

Lipolitic enzymes are secreted by the hepatopancreas or pancreas, and one example is lipase (Zambonino-Infante and Cahu, 2007).

Digestion of proteins, the principal component required for fish growth, is carried out by pancreatic proteases, before the formation of the stomach (Zambonino-Infante and Cahu, 2001). Proteins are cleaved in smaller peptides which are cleaved in aminoacids and dipeptides at brush border. Before the maturation of the brush border, peptides are absorbed and digested in the cytosolic lumen on enterocytes by cytosolic

enzymes, as the leucine-alanine peptidase and acid phosphatase (Zambonino-Infante and Cahu, 2001).

Pepsin is an enzyme whose function is the digestion of proteins. In marine fish larvae stomach only appears several weeks after hatching. In *Pleuronectes americanus* pepsin activity was only detected later on after stomach maturation with the appearance of gastric glands bicarbonate sodium secretion (Douglas *et al.,* 1999). The secretion of pepsin has been related with the transition from larvae to juvenile stage (Zambonino-Infante and Cahu, 2007). The lack of a functional stomach does not hind protein digestion, because there are many other enzymes that digest proteins, such as pancreatic and intestinal enzymes, so this enzyme does not seem crucial for protein digestion (Cahu and Zambonino-Infante, 2001; Zambonino-Infante and Cahu, 2007).

Trypsin is a pancreatic enzyme presenting the typical characteristics of pancreatic enzymes: is expressed at first feeding, the secretion and activity in the intestinal lumen increases in the first weeks of development characterizing the normal maturation process of pancreas. Trypsin is considered an important enzyme for digestion because it is the only pancreatic enzyme that activates other enzymes (Lazo *et al*., 2007). In fishes, trypsin shows a higher affinity for intact proteins than for hydrolysed ones (Zambonino-Infante and Cahu, 2007) and its presence before mouth opening suggests that its activity is not induced by feed (Zambonino-Infante and Cahu, 2001). Besides, studies with Atlantic cod and Atlantic salmon described a strong correlation between trypsin activity and growth rate and food conversion efficiency (Blier *et al*, 2002).

Amylase is another pancreatic enzyme, which can be considered characteristic of the post-natal period, as lactase in mammals. Amylase activity exhibits high levels of activity in the first days of fish larvae development and then shows a decrease in its activity. This decrease may be genetically programmed and reflect different nutritional requirements at different periods of life (Zambonino-Infante and Cahu, 2001). Glycolytic chains and glycogen are components of the diet that can stimulate amylase activity in fish larvae (Cahu and Zambonino-Infante, 2001).

After the digestion by the pancreatic enzymes the components of feed are digested by intestinal enzymes that can be located inside enterocytes (cytosolic) or on brush border membranes. Leucine-alanine peptidase and acid phosphatase are cytosolic enzymes, alkaline phosphatase and aminopeptidase are brush border enzymes. Leucine-alanine peptidase and acid phosphatase are responsible for completing protein digestion, reducing peptides in aminoacids in enterocytes cytoplasm. Micropinocytosis and intracellular digestion of proteins in the posterior

intestine helps in the digestion, compensating the lack of a functional stomach (Cara *et al.*, 2003). These enzymes activity are stimulated by incorporating protein hydrolysate in the diet (Zambonino-Infante and Cahu, 2007).

The concomitant decrease of cytosolic enzymes activity with the increase of brush border enzymes activity is characteristic of intestinal maturation and the beginning of an adult mode of digestion (Zambonino-Infante and Cahu, 2001). It is thought that the structure most probably responsible for the digestion of luminal peptides in vertebrates is the intestinal epithelium. When intestine maturation occurs, peptides digestion is mainly done by alkaline phosphatase and aminopeptidase (Cara *et al.*, 2003). Alkaline phosphatase is sintetized in the Golgi apparatus of the enterocytes (Tengjaroenkul *et al*, 2002).

To summarize, acid phosphatase is an indicator of protein pynocitosis, leucine-alanine and aminopeptidase of complete hydrolysis at an intestinal level and alkaline phosphatase is an indicator of absorption of aminoacids (Cara *et al.*, 2003). Leucine-alanine has been used as a marker of larval mode of digestion (Kvåle *et al.*, 2007).

Maturation of enterocytes is of great importance for the larvae, since good growth and survival is achieved with early maturation of enterocytes. This maturation is genetically programmed but an inadequate diet may delay or avoid the enterocyte maturation inducing larvae mortality (Cahu and Zambonino-Infante, 2001; Zambonino-Infante and Cahu, 2007).

Lipase is one of the main lipolitic enzymes, and is secreted as a response to the presence in the lumen of his substrate of triglycerides. But the secretion mediated by the substrate has limits because 15% of triglycerides in the feed is the maximum capacity of lipase synthesis for some marine fish species (Cahu and Zambonino-Infante, 2001). Lipase is responsible for catalysing the hydrolysis of carboxy-ester bonds. The region of the intestine were occur the digestion and absorption of lipids is the anterior intestine (Zambonino-Infante and Cahu, 2007). In young larvae regulation of lipolitic enzymes seems to be efficient, fact that can explain why larval growth is so dependent on dietary lipid levels (Cahu and Zambonino-Infante, 2001).

The total activity of enzymes increases with age due to an increase in the larval size, on the contrary the specific activity decreases with age, not because the enzymatic activity decrease but because there are an increase in tissue proteins (Zambonino-Infante and Cahu, 2001).

The Species

Diplodus sargus (Lineaus 1758) / White seabream

European aquaculture is developing very fast, and in Mediterranean countries is supported mainly by gilthead seabream (*Sparus aurata*) and sea bass (*Dicentrarchus labrax*) (Aquamedia). Since production of these species has increased, prices are decreasing and industry profits had been severely reduced. For this reason *D. sargus* cultivation assumed a great importance, as new candidate to extend the range of cultivated species (Cara *et al.*, 2003) and assumes a great role in Mediterranean aquaculture because of its high value, demand and easy adaptation to captivity (Sá *et al.*, 2006). Its high potential is partly due to the decrease of more than 50%, in catches, between 1987 and 2004 in the southern of Portugal (D'Anna *et al.*, 2004, Santos *et al.*, 2006).

As the methods of cultivation from gilthead seabream are similar to the ones that can be used with white seabream they were adopted. Although gilthead and white seabream presented a similar growth, white seabream fingerlings grows faster but in later stages the growth rate decrease considerably, compared with gilthead seabream (Sá *et al.*, 2006).

White seabream farming exhibits still several bottlenecks, such as the lack of information on nutritional requirements and consequently the difficulty on formulating the ideal diet for an optimal growth hinder the farming of this species (Sá *et al.*, 2006). One of the main problems is the persistence of skeletal deformities (Saavedra *et al.,* 2006) which is probably a consequence of a non adequate food.

The essential steps of organogenesis are similar in all teleost fish (Sánchez-Amaya *et al*, 2007). Sarasquete *et al.* (1995) studied the larval ontogeny of gilthead seabream, a sparidae fish. At 3 DAH (days after hatching), when gilthead seabream first feed it has the mouth and anus opened, pancreas, liver and a digestive tract differentiated in three portions: foregut including the oesophagus and a primordial stomach, the midgut (anterior intestine) and the hindgut (posterior intestine). Between 4 and 6 DAH the pyloric sphincter and intestinal valves appear. At 5 DAH larvae had lipid vacuoles in the enterocytes of midgut. Around 6-7 DAH acidophilic supranuclear inclusions become visible in the epithelial cells of the hindgut. Gastric glands were not observed in the first 30 days of life of gilthead seabream.

Some researchers pointed out that at early stages this species is omnivorous, eating both algae and small larvae, changing to carnivorous feeding behaviour at an adult stage, when it begins to eat bivalves and others molluscs (Sá *et al.*, 2006). Nonetheless it is suggested that *D. sargus* is omnivorous, having an opportunistic behaviour (Ozorio *et al.*, 2006). More specifically, white seabream is a diurnal omnivorous that have a diet dominated by algae and benthic invertebrates. They eat mostly algae, and also echinodermata, mainly composed by the sea urchin *Arbacia*

lixula, *Sphaerechinus granularis* and *Paracentrotus lividus*. The third group more eaten is Barnacles especially from the family Verrucidae. Worms are frequently eaten too and Gastropods mainly from the sub-classe Prosobranchia, including *Patella sp., Ocinebrina aciculate, Jujubinus pseudogravinae* and *Bittium sp*, are an important group. At lower levels they predate fish eggs, amphipods, gastropods, fish, tunicates, decapods, bivalves and others invertebrates (Figueiredo *et al.,* 2005).

In wild, white seabream usually live in the rocky infralittoral fish assemblage (D'Anna *et al*., 2004). Rocky habitats have shown to give a better environment, giving more food and protection against predation, allowing fish to become twofold fatter when compared with sandy ones (Ozorio *et al*., 2006).

***Solea senegalensis* (Kaup, 1858) / Senegalese sole**

Since the 1990´s sole has been considered a promising specie for aquaculture in Europe (Conceição *et al.,* 2007), because of its high price, high demand and decline in fisheries. It is a common fish species in Mediterranean and southern Atlantic waters, well adapted to warm climates, normaly exploited in southern Europe and are highly economical (Cañavate & Fernández-Díaz, 1999; Dinis et al., 1999).

Sole is a gonochoric species, with the females maturing at age 3+ when the total length is 32 cm, spawning in spring, between March-June. In captivity these species have natural spawning, stopping the egg emission below 16 ºC (Dinis *et al*., 1999). Adult fish can be infected by the ectoparasite *Hemibdella solea* and have outbreaks of pasteurellosis or pseudotuberculosis (Dinis *et al*., 1999).

Sole postlarvae suffers a drastic metamorphosis some days after hatching, that starts on 11 DAH and is completed by 19 DAH, settling in the bottom of the rearing tanks (Dinis et al., 1999). Sole presents a peculiar mode of ingestion, being not an active predator, waits that feed sinks and eat in the bottom of tanks, grazing continuously on *Artemia sp*. (Engrola *et al.,* 2007). Sole can be successfully first feed with Artemia nauplii or rotifers being the last one the prey most used (Dinis et al., 1999).

Besides the complex metamorphosis sole as other problems of cultivation such as difficulties in weaning, occasional problems of malpigmentation, high incidence of skeletal deformities and morphological malformations related with the migration of the eye (Dinis *et al*., 1999; Conceição *et al.,* 2007). Juveniles can suffer for example from epizootic mortalities due to pasteurellosis cause by the presence of *Photobacterium damsela* subsp. *piscicida* (Dinis et al., 1999).

Major morphological changes were observed within the first 2 days of sole life. At 2 DAH, when sole first feed it has the mouth and anus opened, pancreas, liver and the

digestive tract is differentiated in five portions: buccal-pharyngeal cavity, oesophagus, incipient stomach, anterior and posterior intestine. At the same age in the anterior portion of intestine it can be observed a brush border which is a more basophilic cytoplasm with several apical vesicles and dense granules. The thickness of the brush border can be observed with larval growth. Around 16 DAH when larvae start to lose symmetry the anterior intestine delineates the lower part of the abdominal cavity and posterior intestine delineated the posterior part of the cavity. Around 27 DAH gastric glands are seen for the first time (Ribeiro *et al.,* 1999b).

In the wild the feeding regime is based in majority in polychaetes (*Hediste diversicolor*) and in less quantities amphipods, copepods and isopods (Dinis *et al*., 1999; Sá *et al*., 2003).

Objectives of this study

The present study aimed to study the influence of inert diets on the digestive capacity of fish larvae.

In the case of *Diplodus sargus* two sub-objectives were defined, first to study the pattern of digestive enzymes during larval development, and secondly to analyse the effect of an early weaning on larval digestive capacity.

Regarding *Solea senegalensis* it was aimed to study different weaning strategies, namely sudden weaning and co-feeding, on sole digestive capacity.

2. Materials and methods

2.1 Larval rearing conditions

Larval rearing was carried out at Estação Piloto de Piscicultura de Olhão (EPPO) from INRB (Instituto Nacional dos Recursos Biológicos) I.P. (Instituto público) –IPIMAR for both fish species, in 300 L cylindrical round bottom tanks maintained in a semi-closed circuit, in a temperature controlled room.

2.1.2 Broodstock and egg incubation

D. sargus

White seabream broodstock composed of 30 fishes with an average weight and length of 999.5 g and 37.5 cm respectively, were captured from the wild and stocked in 10.6 m^3 tanks. Broodstock were kept at a density of 2.8 kg/m^3 and a biomass of 29.9 kg. Fish were fed daily with squid (*Illex spp.*) and inert diet "RodoSoja® Ultra Energia, (Sorgal®", Portugal) with a size of 8 to 10 mm. They were submitted to a 9h light: 15h dark photoperiod and water temperature was kept at 18 ± 1 ºC, during the spawning season.

Eggs were obtained from natural spawning and immediately collected in an egg-collector. Eggs were transferred to a glass beacker to separated, viable buoyant eggs from the dead sinking eggs. The spawn weighted 332 g and exhibited a hatching percentage of 26%.

Eggs were incubated in 220 l incubators with a maximum density of 1800 eggs L^{-1} with a seawater flow of 6 L h^{-1} and a temperature of 18 ± 1 ºC. Oxygen saturation was always close to saturation (100%) and salinity was maintained at 35 ‰.

S. senegalensis

Senegalese sole broodstock was composed by 45 fishes with an average weight and length of 1500 g and 50 cm respectively, these fishes were captured from the wild and stocked in 18 m^3 tank with a seawater supply of 20 L min^{-1}. Broodstock were kept at a density of 3.75 kg /m^2 and a biomass of 67.5 kg. Fish were fed daily with semi-dry diet Alpis® for broodstock. They were subjected to natural photoperiod and water temperature was kept at 13-19 ºC.

Eggs were obtained from natural spawning and immediately collected in an egg-collector. Eggs were transferred to a glass beacker to separated, viable buoyant eggs

from the dead sinking eggs. The spawn weighted 223 g and exhibited a hatching percentage of 90%.

Eggs were incubated in 220 l incubators with a maximum density of 1100 eggs L^{-1} with a seawater flow of 6 L h^{-1} and a temperature of 18 ± 1 ºC. Oxygen saturation was always close to saturation (100%) and salinity was maintained at 35 ‰.

2.1.3 Larval rearing

D. sargus

At 2 DAH white seabream larvae were transferred to a semi-closed sea water system, in 300 L cylindrical round bottom tanks at a density of 80 individuals L^{-1}. On a daily basis, tanks were cleaned, dead larvae collected and water temperature, dissolved oxygen and salinity were monitored. During the rearing period temperature varied between 17.5-19.7 ºC, oxygen between 6.5-9.2 ppm and salinity between 34-36 ‰. During the first 7 days after hatching (DAH) the water flow was 0.4 L min^{-1}, afterwards the flow was increased with larvae age reaching 2 L min^{-1} by the end of the experiment. Water flow out the tanks by draining through an 80 µm mesh size during the day and a 150 µm mesh size during the night. When larvae were bigger a 500 µm mesh size was used during 24 h. The different mesh sizes were needed due to the different size of the live prey offered to larvae, thus avoiding that prey pass through the mesh before the larvae eat them. Before re-entering in the tanks water had pass through a mechanical filter to remove solid particles, a biological filter which removed solid particles too, and a UV light filter for disinfection. Photoperiod was 14 h light and 10 h dark during all the rearing period.

Fish larvae were fed rotifers (*Brachionus sp*) enriched with Protein Selco® from 4 DAH until 26 DAH, at a quantity that varied between 6,000,000 and 600,000 individuals per tank respectively at the first and last days. *Nannochloropsis oculata* and *Isochrysis galbana*, at a quantity of 300,000 cells L^{-1}, were used as green-water technique during the period larval fish were fed with rotifers. Fish larvae were fed *Artemia sp* AF ("*Artemia franciscana* grade") nauplii between 13 and 19 DAH, respectively with 4,500 and 75,000 individuals per tank. The amount of nauplii was low in the first days to allow larvae to adapt to the new kind of feed. This amount increased until *Artemia sp* AF was replaced by *Artemia sp* EG ("*Artemia sp* enrichment grade"). At 16 DAH larvae begun to be fed with *Artemia sp* EG metanauplii in a quantity that varied between 60,000 and 2,400 individuals per tank respectively at the first and last days of feeding with metanauplii. Both artemia were enriched with Rich Advanced®.

White seabream larvae feeding plan, before the beginning of treatments is presented in Fig. 1.

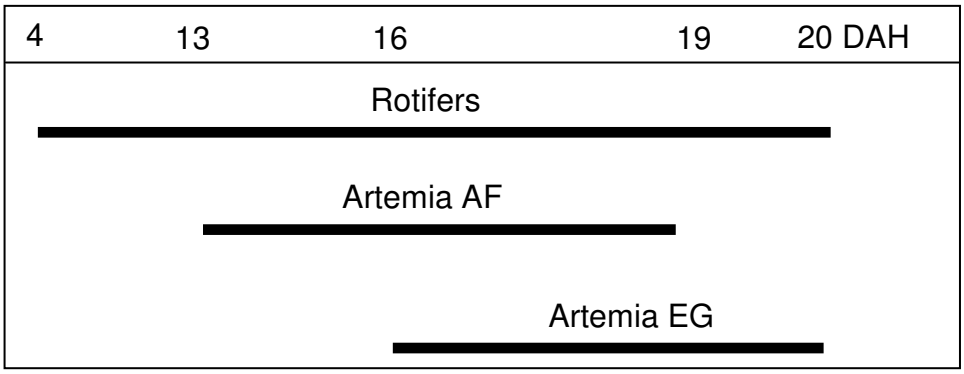

Fig. 1 – White seabream larvae feeding plan, before the beginning of treatments.

S. senegalensis

At 2 DAH sole larvae were transferred to a semi-closed sea water system, in 300 L cylindrical round bottom tanks at a density of 40 individuals L^{-1}. On a daily basis, tanks were cleaned, dead larvae collected and water temperature, dissolved oxygen and salinity were monitored. During the rearing period temperature were kept at 20 ± 1 ºC, oxygen between 70-100 % and salinity between 36-39 ‰. Water flow during the experiment was kept at 0.8 L min^{-1}. Water replacement (flow out and flow in) was done as reported previously for *D. sargus*. Photoperiod was 16 h light and 8 h dark during all the rearing period.

Fish larvae were fed rotifers (*Brachionus sp*) enriched with microalgae (*Nannochloropsis oculata*) from 2 DAH until 12 DAH, at a quantity that varied between 4,500,000 and 1,500,000 individuals per tank respectively at the first and last days. *Nannochloropsis oculata* at a density of 300,000 cells L^{-1} was used as green-water technique during the period larval fish were fed with rotifers. Fish larvae were fed with *Artemia sp* EG metanauplii between 8 and 29 DAH, at a quantity that varied between 150,000 and 900,000 individuals mL^{-1}. The amount of metanauplii was low in the first days to allow larvae to adapt to the new kind of feed. Artemia was enriched with microalgae such as rotifers. From 8 to 19 DAH metanauplii were given alive, whereas from 20 to 29 DAH it was given frozen.

Sole larvae feeding plan, before the beginning of treatments is presented in Fig. 2.

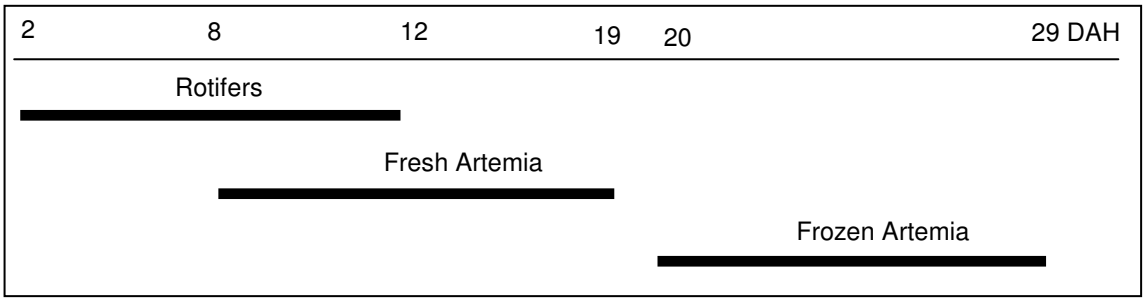

Fig. 2 – Sole larvae feeding plan, before the beginning of treatments.

2.1.3.1 Experimental design

D. sargus

The objective of the white seabream experiment was to analyse the effect of early weaning on digestive capacity of larvae. Therefore in treatment W20 inert diet was introduced when fish larvae were 20 DAH whereas in treatment W27 inert diet was introduced at 27 DAH. Weaning was realised in a co-feeding regime with live feed for 6 days. Fish were cultivated until 3 weeks after inert diet introduction in the feeding regime.

In treatment W20 larvae were co-fed with inert diet, rotifers and Artemia EG which densities decreased into zero by the end of the co-feeding period. There were used 3 types of inert diet, Nippai – Ambrose 100® which was given from 20 DAH until 26 DAH, Lucky Star® 1 given from 24 DAH until 41 DAH and Lucky Star® 2 given from 28 DAH until the end of the experiment.

In treatment W27 when larvae started eating inert diet the period of feeding with rotifers was ended, so larvae were co-fed only with inert diet and Artemia EG whose density in the period of co-feeding with inert diet was reduced until stopped. There were used 3 types of inert diet, Nippai – Ambrose 100® which was given from 27 DAH until 29 DAH, Lucky Star® 1 given from 28 DAH until 44 DAH and Lucky Star® 2 given from 30 DAH until the end of the experiment.

Composition, ingredients and size of the food particle of the Lucky Star is presented in table 1.

Larva feeding plan for both treatments was summarized in Fig. 3.

Table 1 – Composition of inert diet given to larvae. The ingredients of the feed are: fish meal, squid meal, cereal, krill meal, yeast, vitamin and minerals. Lucky star 1 feed particle size has 100~200 microns and Lucky star 2 has 200~400 microns.
Source: Manufacture label.

Feed	Composition	% of diet
Lucky Star 1 & 2	Crude Protein	56
	Crude Fat	8
	Crude Fiber	1,4
	Crude Ash	13
	Moisture	10

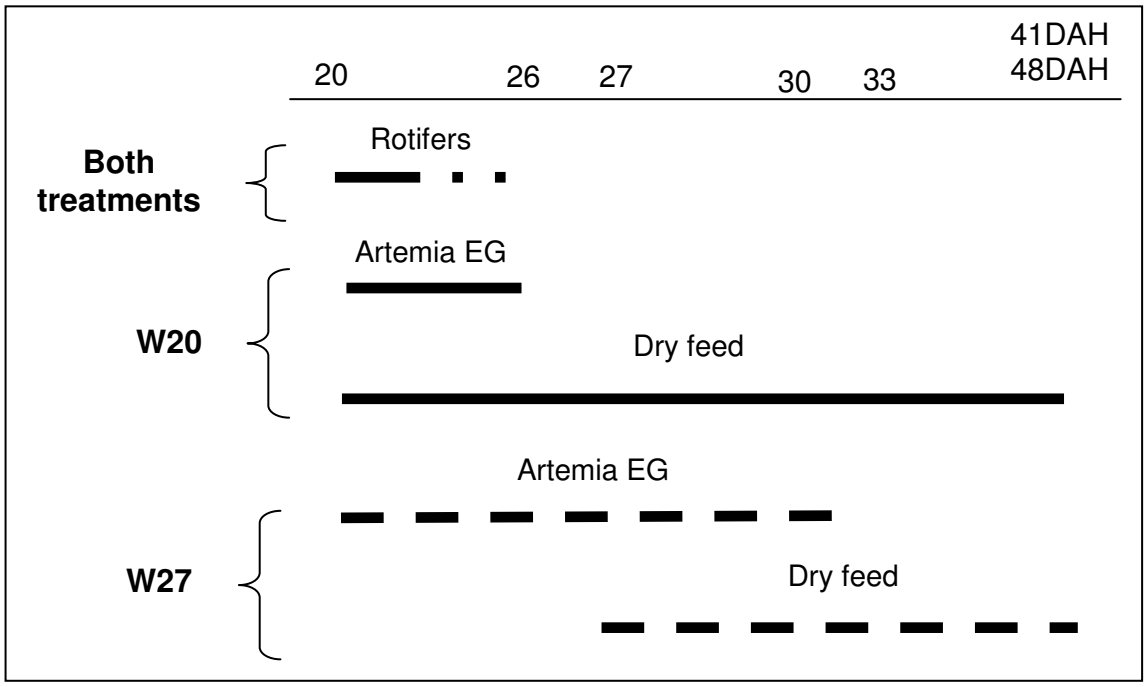

Fig. 3 – White bream larvae feeding plan: Both treatments (semi-dashed line); Treatment W20 (solid line); Treatment W27 (dashed line).

S. senegalensis

The objective of sole experiment was to analyse the effect of two strategies of weaning on digestive capacity, the sudden weaning and co-feeding. Therefore in fish post larvae from Treatment CF diet was introduced at 30 DAH in a co-feeding regime until 34 DAH, whereas in post larvae from Treatment SW diet was introduced at 30 DAH suddenly.

Fish larvae were counted and distributed by six tanks, 3 for each treatment. 1550 individuals were distributed by each experimental tank at the start of experiment. Tanks

were similar to the larval rearing tanks and water conditions were the same as reported earlier.

Fish were fed with AgloNorse® that was given in both treatments until visual satiety. Composition per kg of feed, ingredients and size of the food particle of the inert diet is presented in table 2.

Post larva feeding plan for both treatments was summarized in Fig. 4.

Table 2 – Composition per kg of feed of the inert diet given to post larvae. The ingredients of the feed are: fish meal, fish oil, vegetable oil, carbohydrate, mineral and vitamins. AgloNorse feed particle size has 100~200 microns.
Source: Manufacture label.

Feed	Composition	% of diet
AgloNorse	Protein	59%
	Total fat	21%
	Ash	10%
	Fiber	1%
	Vitamin D	5 iu/g
	Vitamin E	240 mg/kg
	Copper	5 mg/kg

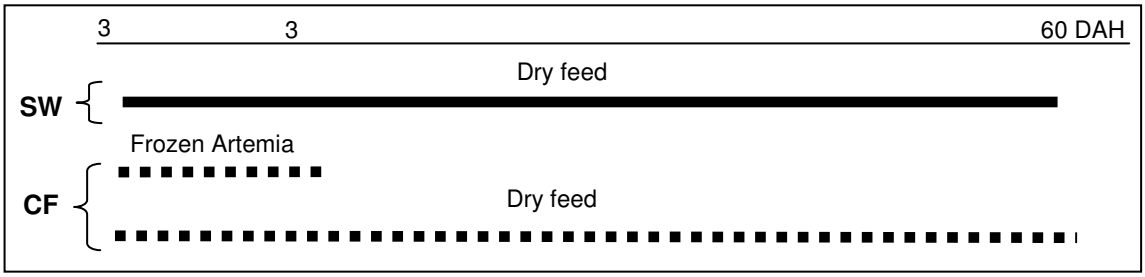

Fig. 4 – Sole larvae feeding plan: Treatment SW (solid line); Treatment CF (dashed line).

2.1.4 Sampling

Sampling was always done before feed distribution.

Larvae for enzymatic analysis were washed with distilled water and frozen in liquid nitrogen (-196 ºC).

Larvae for weight measurement were used first for length measurement and then rinsed with 3 % ammonium formiate and distilled water and then frozen in liquid nitrogen.

At the end of the experiment, larval survival was determined by counting larvae remaining in the tanks.

D. sargus

In order to measure growth in length and weight, 4 groups of 5 larvae were sampled randomly from the 6 tanks in days 0, 2, 9, 13 and 20 DAH. For the same measurements 10 larvae from each tank were sampled randomly and individually in days 27, 34, 41 DAH for both treatments and in 48 DAH only for treatment W27, because the objective was to evaluate growth during the next 3 weeks after the beginning of ingestion of inert diet.

For enzymatic analysis groups of 120 larvae were collected in 0 and 2 DAH, before they were stocked in the rearing tanks. At 9, 13 and 20 DAH 75 larvae were collected. Fish larvae were sampled for each treatment at the introduction of the inert diet and 1 and 3 weeks after. So at 27 DAH 100 larvae were collected and in the posterior ages were collected only 50 larvae.

S. senegalensis

In order to measure growth in length and weight, 24 larvae were sampled at 30 DAH before the beginning of the treatments, after this age 10 larvae were sampled randomly from all tanks in days 35, 40, 45, 50, 55 and 60 DAH.

For enzymatic analysis 3 groups of 60 larvae were collected in 30 DAH before the redistribution by the twelve tanks. At 35 and 40 DAH 80 larvae were collected from each tank, and 60 larvae at 50 and 60 DAH.

2.2 Biometric analytical measurement

Larval length was measured using a binocular microscope with a micrometric eyepiece with a precision of ± 0.05 mm. Length was measured from the tip of the snout to the end of the caudal fin (total length).

Weight was determined on fish larvae freeze-dried for 24 h and then weighted in a high precision balance Sartorius microbalance (1 ± 0.001 mg).

Relative growth rate was calculated by using the formula: RGR (%) = [exp((ln final weight - ln initial weight)/(final day-initial day))-1*100] (Ricker 1958).

2.3 Enzymatic analysis

2.3.1 Fish larvae dissection

D. sargus

Whole body homogenates were used for enzymatic analysis in larvae younger than 20 DAH due to the small size of larvae. After this age, older larvae were dissected to obtain the abdominal cavity. In Fig. 5 is shown the dissection cut lines to obtain the abdominal cavity.

Larvae were slightly defrost to allow individualization and dissected on a glass maintained on ice (0 ºC) to preserve enzymes activity. The portion obtained was weighted in a balance (Precisa 100A-300M (1 ± 0.00001 g)) to calculate the homogenization volume.

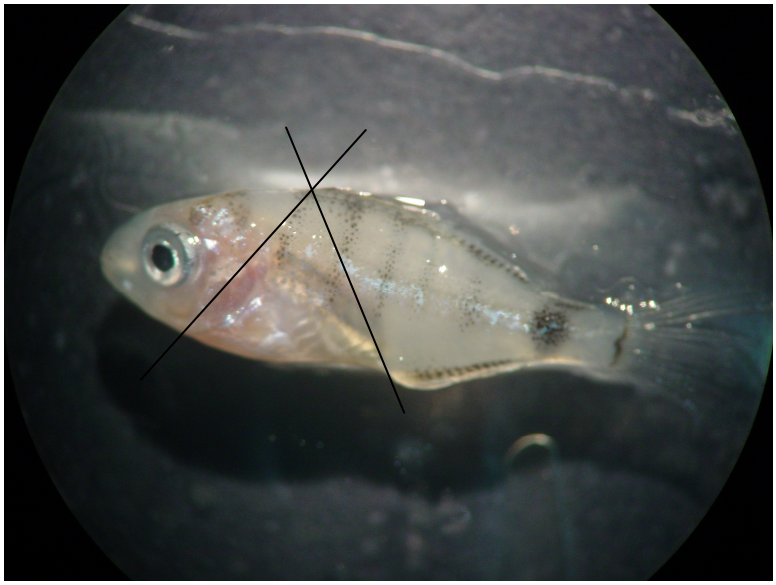

Fig. 5 – Lines indicate dissection cut zones to obtain the abdominal cavity in *D. Sargus.*

S. senegalensis

Larvae with 30, 35 and 40 DAH were dissected to obtain the abdominal cavity. Larvae with 50 and 60 DAH were dissected to obtain the pancreatic and intestinal segment. Digestive tracts were cut at the junction of the oesophagus and at the pyloric sphincter to obtain the pancreatic segment including pancreas, stomach and liver, and intestinal segment (Ribeiro *et al.,* 2002). In Fig. 6 is shown the pancreatic and intestinal segment and the area of cut. Larvae were slightly defrost to allow individualization and dissected on a glass maintained on ice (0 ºC) to preserve enzymes activity. The portions obtained were weighted in a balance (Precisa 100A-300M (1 ± 0.00001 g)) to calculate the homogenization volume.

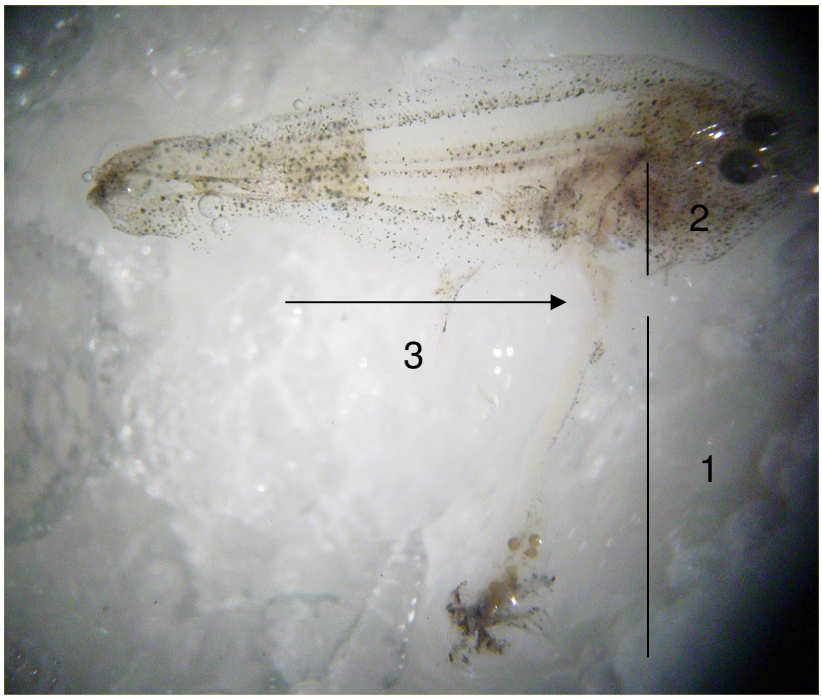

Fig. 6 – Different larvae segments: 1- Intestinal segment; 2- Pancreatic segment; 3- Cut zone to individualize intestinal from pancreatic segment.

D. sargus* and *S. senegalensis

The pancreatic segment (*S. senegalensis*) and whole larval body (*D. sargus*) were homogenized in 15 volumes (w/v) of ice cold distilled water for periods of 2 x 30 seconds. Samples were centrifuged at 3300g at 4 ºC for 3 minutes, and supernatant was sonicated for 10 seconds. For the purification of the brush border segments were homogenized in 30 volumes (w/v) of ice cold Manitol-Tris during 2 x 30 seconds. Afterwards a volume of this homogenate was used for brush border purification whereas the other volume of intestinal homogenate was submitted to the method previously described for pancreas. To the homogenate volume used for brush border purification a certain volume of $CaCl_2$ (0,1M) was added to obtain 10 mM, in order to preserve membrane stability. Samples were then centrifuged at 9000g at 4 ºC for 10 minutes, and the supernatant was centrifuged at 34000g at 4 ºC for 20 minutes. The supernatant was discarded and the precipitated ressuspended with DTT (1mM).

2.3.2 Enzymatic assays

Table 3 – Enzymes assayed and in which species and segment were assayed.

Enzyme	Detection site	Species
Trypsin	pancreas, intestine	*S. senegalensis, D. sargus*
Amylase	pancreas, intestine	*S. senegalensis, D. sargus*
Pepsin	pancreas	*S. senegalensis, D. sargus*
Lipase	pancreas, intestine	*D. sargus*
Aminopeptidase	intestine, brush border	*S. senegalensis, D. sargus*
Alkaline phosphatase	intestine, brush border	*S. senegalensis, D. sargus*
Acid phosphatase	intestine	*D. sargus*
Leucine-alanine peptidase	intestine	*S. senegalensis, D. sargus*

2.3.2.1 Trypsin analysis

Trypsin activity determination was based on the study by Holm *et al.* 1988. In a cuvette, 1000 µl of buffer trizma-$CaCl_2$ (20 mM) pH 8.2, 10 µl of Bapna 0.1 M (Nα-Benzoyl-DL-arginine-*p*-nitroanilide) and 50 µl of homogenate were mixed. All processes were performed at 25 ºC. The reaction was read in a spectrophotometer at 407 nm.

2.3.2.2 Aminopeptidase analysis

Aminopeptidase activity determination was based on the study by Maroux *et al.* 1973. In a cuvette 2.47 ml of buffer phosphate (80 mM), 50 µl of the substrate L-leucine p-nitroanilide (0.1 M) and 50 µl of the homogenate were mixed. All processes were performed at 37 ºC. The reaction was read in a spectrophotometer at 410 nm.

2.3.2.3 Amylase analysis

Amylase activity was measured using starch as the substrate (Métais & Bieth, 1968). In a reaction tube 100 µl of starch solution (0.07 M) and 50 µl of the homogenate were incubated in a water bath at 37 ºC during 30 minutes. At the end of this period 20 µl of HCl 1M was added to stop the reaction. Then to the reaction tube were added 400 µl of distilled water and 2 ml of iodine solution. A similar procedure was carried out for the blank tube except that the 50 µl of the homogenate was added only after the reaction was stopped with HCl. The colour developed in these tubes was read against the iodine solution at 580 nm.

2.3.2.4 Alkaline phosphatase analysis

Alkaline phosphatase determination was based on the study by Bessey *et al*. 1946. In a cuvette, 2.53 ml of a substrate pNPP 5 mM (*p*-nitrophenylphosphate) dissolved in a solution of carbonate buffer (30 mM) and 50 µl of homogenate were mixed. All processes were performed at 37 ºC. The reaction was read in a spectrophotometer at 407 nm.

2.3.2.5 Pepsin analysis

Pepsin activity was measured using haemoglobin 2.5%, pH 2 as the substrate (Anson, 1938). The substrate was prepared with haemoglobin, water and HCl 1M. In a reaction tube 500 µl of substrate was incubated in a water bath at 37 ºC during 5 minutes. At the end of this period 100 µl of homogenate was added and incubated again during 20 minutes. At the end of this period 1000 µl of TCA 5 % was added to stop the reaction. After stopping the reaction the tube was again incubated during 5 minutes. A similar procedure was carried out for the blank tube except that the 100 µl of the homogenate were added only after the reaction was stopped with TCA. The reaction and blank tube were centrifuged at 4000 g, 4 ºC during 6 minutes, and read immediately at 280 nm against deionised water.

2.3.2.6 Lipase analysis

The activity was measured using p-nitrophenyl myristate as a substrate based on the study by Iijima *et al*., 1998. The substrate was prepared using Tris-HCl buffer (0.25 M, pH 9), 2-methoxyethanol (0.25 mM), p-nitrophenyl myristate (0.53 mM) and sodium cholate (5 mM). Reaction was stopped using a solution of acetone and n-heptane (1:1). In a reaction tube 500 µl of substrate and 10 µl of homogenate were incubated at 30 ºC during 15 minutes. At the end of this period 700 µl of a stop solution was added to stop the reaction. A similar procedure was carried out for the blank tube except that the 10 µl of the homogenate was added only after the reaction was stopped with the stop solution.

Before reading at 405 nm in a spectrophotometer the blank and reaction tubes were centrifuged at 6080 g, 4 ºC during 2 minutes. The blank and reaction were read against a zero solution that was prepared like the blank but instead of homogenate in the end was added 10 µl of distilled water.

2.3.2.7 Intestinal peptidases analysis

Intestinal peptidase, leucine-alanine peptidase, determination was based on the study by Nicholson & Kim (1975) using leucine-alanine 0.01 M as substrate. The

reaction tube with homogenate was done with 0.5 ml of substrate, 1 ml of LAOR (L-amino oxidase, horseradish peroxidase, o-dianisine and was dissolved in a Tris-HCl 50 mM buffer), 5 minutes in a bath at 37 ºC, 25 µl of homogenate, 20 minutes bath and the reaction was stopped with sulphuric acid. The blank sample tube was made in the same way but instead of adding substrate was added 0.5 ml of Tris-HCl buffer. The blank substrate tube was made the same way as the reaction tube but instead of homogenate 25 µl of water was added. The blank zero was made with only Tris-HCl buffer, LAOR and sulphuric acid to stop the reaction. A standard curve using leucine (0.01 M) was done to determine this enzyme activity. All tubes were read at 530 nm in the spectrophotometer against the blank zero.

2.3.2.8 Acid phosphatase analysis

Acid phosphatase determination was based on the study by Terra *et al.* 1979. In a cuvette, 2.5 ml of a substrate pNPP 5.5 mM (*p*-nitrophenylphosphate) made in a solution of citrate buffer 0.1 M (citric acid and sodium citrate) and 50 µl of homogenate were mixed. All processes were performed at 37 ºC. The reaction was read in a spectrophotometer at 405 nm during 5 minutes.

2.3.2.9 Protein analysis

Protein was quantified using the Bradford method (Bradford, 1976) for the pancreatic, intestinal homogenate and brush border homogenate. Depending on the tissue analysed a variable amount of homogenate was mix with the Bradford solution (coumassie blue, ethanol, orthophosphoric acid and distilled water) for 2 minutes and read in a spectrophotometer at 595 nm.

2.3.2.10 Enzyme activity expression

Enzyme activities were calculated as micromoles of substrate hydrolysed per minute (i.e., U) at 37 ºC for alkaline phosphatase, aminopeptidase and leucine-alanine, and 25 ºC for trypsin. Amylase activity was expressed as the equivalent enzyme activity which was required to hydrolyse 1 mg of starch in 30 minutes at 37 ºC. Pepsin activity was expressed as specific activity with 1 U representing 1 mM equivalent of tyrosine liberated per minute per mg of protein at 37 ºC. One unit of lipase activity was defined as 1 mmoL of p-nitrophenol released per minute at 30 ºC.

Enzyme activities were expressed as specific activities, i.e., U/mg protein or mU/mg protein, and as segmental activities, i.e., total activity per larvae segment.

As larvae (*D. sargus*) from the different treatments had inert diet introduced at different ages it was determined the relative increase using the formula: RI = final total activity (week 1 and 3) / initial total activity (week 0).

Secretion of pancreatic enzymes, trypsin and amylase, was calculated as the ratio of the total activity in the intestinal segment, over the sum of total activity in pancreas and intestine for each enzyme (Zambonino-Infante and Cahu 1999).

The enterocyte maturation index was determined as the ratio between brush border enzyme total activity (alkaline phosphatase, aminopeptidase) and leucine-alanine peptidase total activity (Zambonino-Infante *et al.*, 1997).

2.4 Data analysis

All data of dry weight (mg), total length (mm), specific activity and total activity of digestive enzymes were expressed as means ± standard deviation (SD) of treatment replicates.

Homogeneity of variance was verified using Bartlett's test. Enzymatic activity data between ages were compared by one-way ANOVA, both for *S. senegalensis* and for *D. sargus*, being followed by Tukey-Kramer Multiple comparison test, when significant differences were found at the $\alpha = 0.05$ level (GraphPad InStat 3 (GraphPad software, California, U.S.A.)).

Enzymatic activity data both for *S. senegalensis* and *D. sargus* treatments were compared by Unpaired t test. Differences were considered significant when $P < 0.05$ (GraphPad InStat 3 (GraphPad software, California, U.S.A.)).

Data for *S. senegalensis* were separately compared between 30 and 40 DAH and at 50 and 60 DAH. Similarly data for *D. sargus* were separately compared between 0 and 20 DAH and between treatments.

3. Results

3.1 Growth and survival

D. sargus

Ontogenetic development

Two periods of growth could be identified during white seabream development (Fig. 7). A first period with a smooth increase until 9 DAH, and after this a more pronounced growth specially in term of weight.

The relative growth rate (Table 4), presented an overall increase during larval development, except between 2 and 9 DAH where larvae presented the lower growth rate.

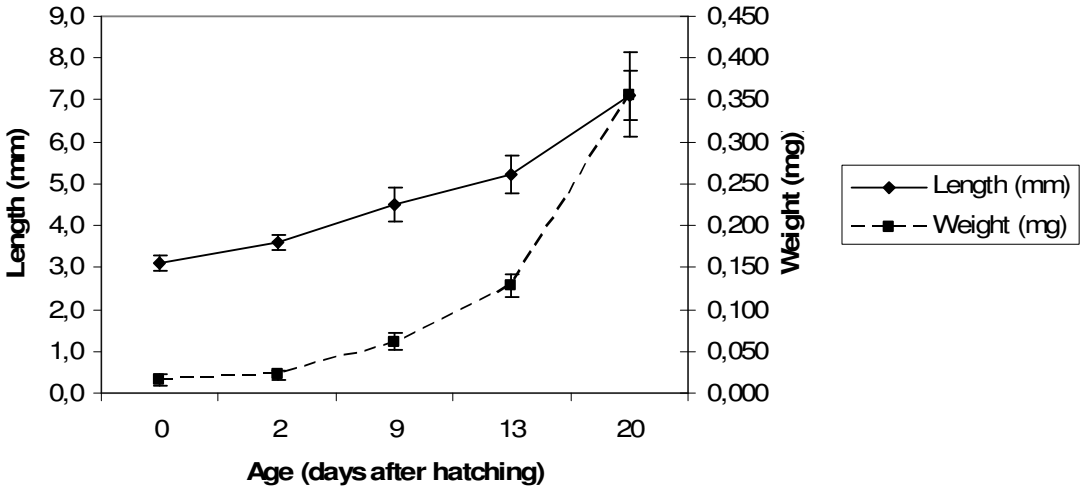

Fig. 7 – Growth of *Diplodus sargus* larvae: total length (solid line); dry weight (dashed line). Values are presented as means ± standard deviation (n = 20).

Table 4 – Relative Growth Rate (RGR) (% day^{-1}) during ontogenetic development of *Diplodus sargus*, using dry weight.

Ontogenesis	
0-2 DAH	18,93
2-9 DAH	16,13
9-13 DAH	20,29
13-20 DAH	29,08

Growth after weaning

Growth was slightly higher in larvae from W27 treatment being both length (Fig. 8) and weight (Fig. 9) statistically superior at 34 and 41 DAH.

In terms of relative growth rate (Table 5), at week 0-1 larvae from W27 treatment presented a higher RGR, while at week 1-3 larvae from W20 treatment had a higher RGR.

Survival rate was calculated as 3.3% in W20 treatment and 3.8% in W27.

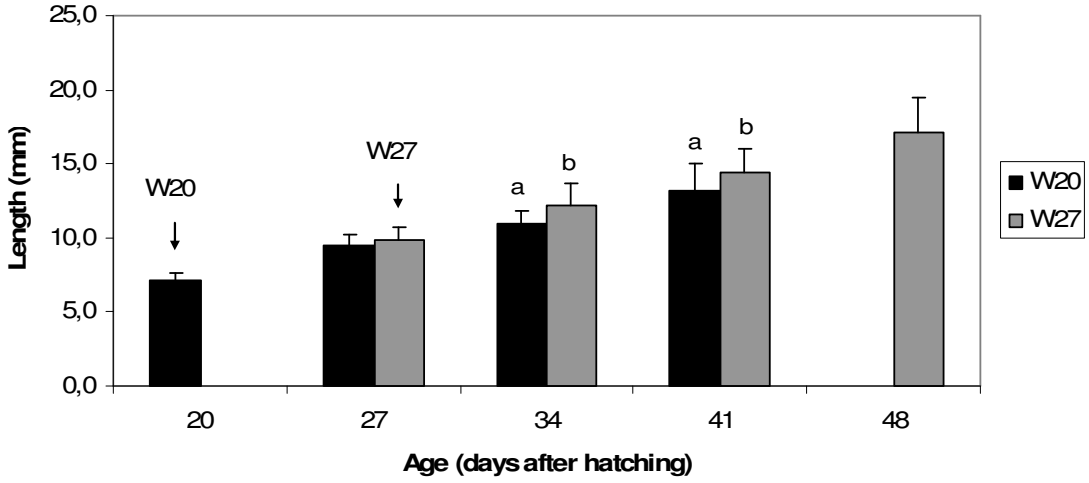

Fig. 8 – Total length growth of *Dipodus sargus* larvae under the W20 and W27 treatments. Values are presented as means ± standard deviation (n = 30). Different superscripts indicate significant differences between means. Arrows indicate the day of weaning for each treatment.

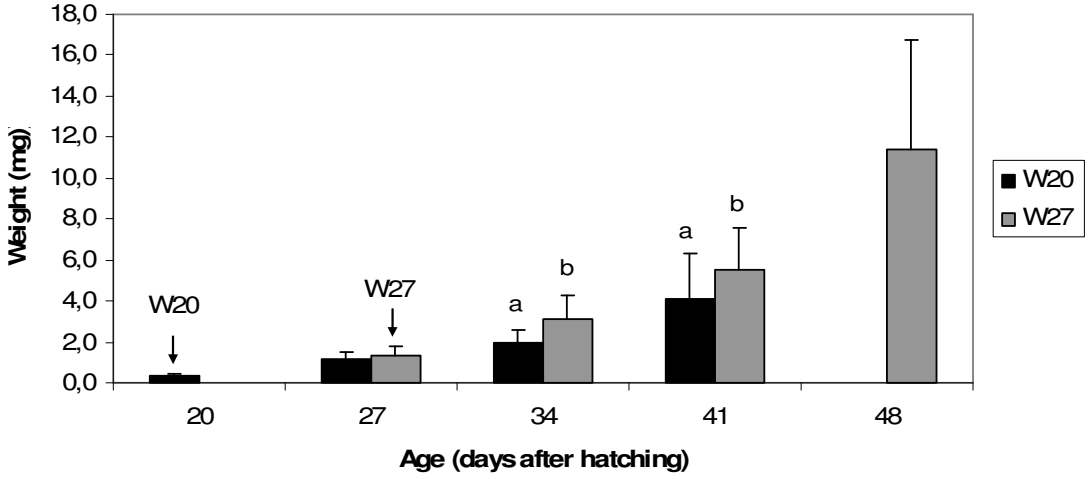

Fig. 9 – Dry weight growth of *Diplodus sargus* larvae under the W20 and W27 treatments. Values are presented as means ± standard deviation (n = 30). Different superscripts indicate significant differences between means. Arrows indicate the day of weaning for each treatment.

Table 5 – Relative Growth Rate (RGR) (% day^{-1}) after weaning of *Diplodus sargus*, using dry weight.

Weeks after weaning	RGR (W20)	RGR (W27)
0*	12,75	12,75
0-1	7,35	8,54
1-3	11,17	10,96

* To calculate RGR in the week of inert diet introduction was used the age before weaning and the age at weaning.

S. senegalensis

Sole post larvae length (Fig. 10) was more or less constant, throughout the experiment in both experiments. Length of post larvae from treatment SW at 60 DAH was 1.1 times higher than post larvae from CF treatment.

Sole post larvae dry weight (Fig. 11) exhibited more dispersion than length. In fact post larvae from SW treatment had a significantly higher weight at 40 and 60 DAH than post larvae from CF treatment, while at 45 DAH an opposite pattern was observed.

Relative growth rate exhibited fluctuations during post larvae development presenting periods where fish did not growth. Fish post larvae from CF treatment exhibited the higher growth rate between 40-45 DAH and post larvae from SW treatment between 50-55 DAH. The RGR during the experimental period (30-60 DAH) was higher in post larvae from SW treatment than in post larvae from CF treatment.

Survival rate was calculated as 22% in treatment CF and 25% in SW.

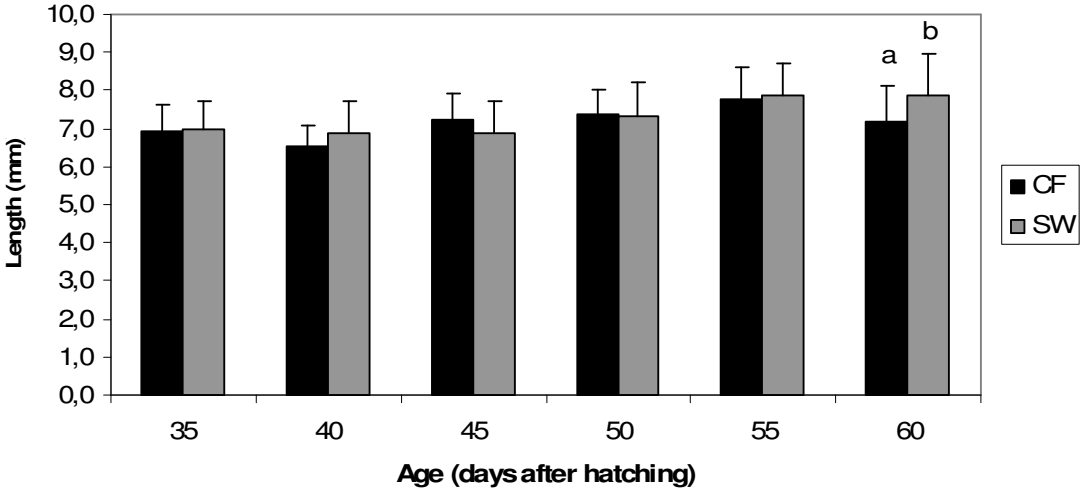

Fig. 10 – Total length growth of *Solea senegalensis* post larvae under the CF (co-feeding) and SW (sudden weaning) treatments. Values are presented as means ± standard deviation (n = 30). Different superscripts indicate significant differences between means.

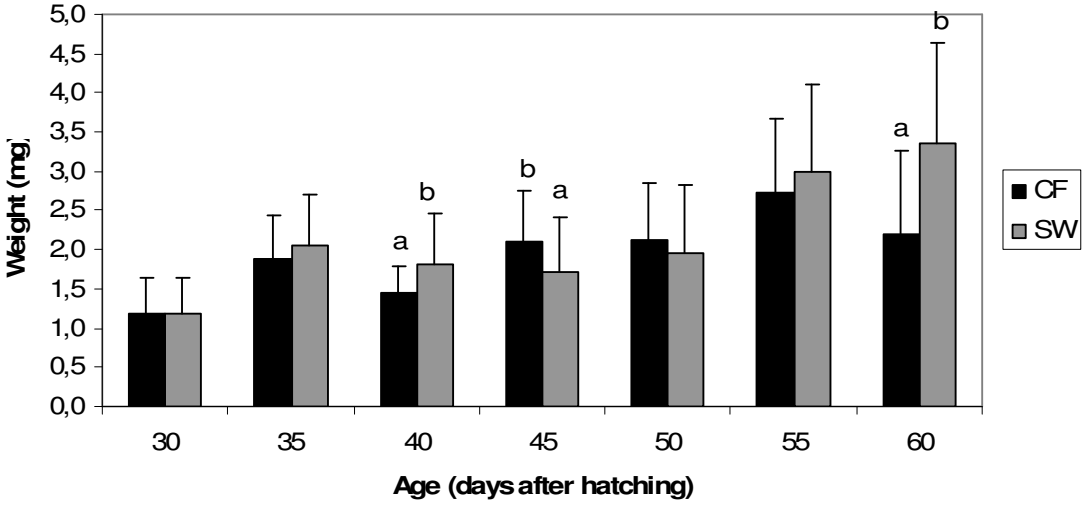

Fig. 11 – Dry weight growth of *Solea senegalensis* post larvae under the CF (co-feeding) and SW (sudden weaning) treatments. Values are presented as means ± standard deviation (n = 30). Different superscripts indicate significant differences between means.

Table 6 – Relative Growth Rate (RGR) (% day^{-1}) in the different growth periods, using dry weight.

	Relative Growth Rate (RGR)	
Growth period (DAH)	**CF**	**SW**
30-40	-2,53	-1,22
40-45	7,62	-0,95
45-50	0,38	2,49
50-55	5,03	8,87
55-60	-4,30	2,32
30-60	0,52	1,65

3.2 Enzymatic activity

D. sargus

Enzymatic ontogenetic development

Trypsin and amylase (Fig. 12) were detected at hatching. Trypsin specific activity exhibited a slight decreased in its activity until 9 DAH, followed by an increase until 20 DAH. Trypsin specific activity at 13 and 20 DAH was more than 2 times higher than at earlier ages.

Amylase specific activity exhibited a slight increase at 2 DAH followed by a slightly decrease at 9 DAH to a value that was maintained rather stable until 20 DAH.

Pepsin activity (Fig. 12) was detected at hatching being the highest level of activity observed when compared with the other ages analysed. At 2 DAH the activity was not detected increasing after this age until 20 DAH.

Lipase activity (Fig. 12) was only detected at 9 DAH followed by a slight increase until 13 DAH to a value that was maintained until 20 DAH.

Alkaline phosphatase, aminopeptidase and acid phosphatase (Fig. 12) were detected as early as hatching.

Alkaline phosphatase specific activity of white seabream fish larvae, increased until 20 DAH. Except for 9 and 13 DAH all other ages differ significantly from each other.

Aminopeptidase specific activity kept a more or less constant level of activity until 9 DAH, increasing afterwards until 20 DAH, presenting a value 2.25 times higher than at 0, 2 and 9 DAH.

Acid phosphatase specific activity, kept the level of activity relatively constant until 9 DAH, after this age the level of activity increased until 20 DAH. Acid phosphatase specific activity of fish aged 13 and 20 DAH differ significantly from other ages and from each other.

Leucine-alanine specific activity (Fig. 12) was only detected at 9 DAH and increased significantly at 13 DAH, keeping a constant level until 20 DAH. The specific activity at 9 DAH is 2.2 times lower than at 13 and 20 DAH.

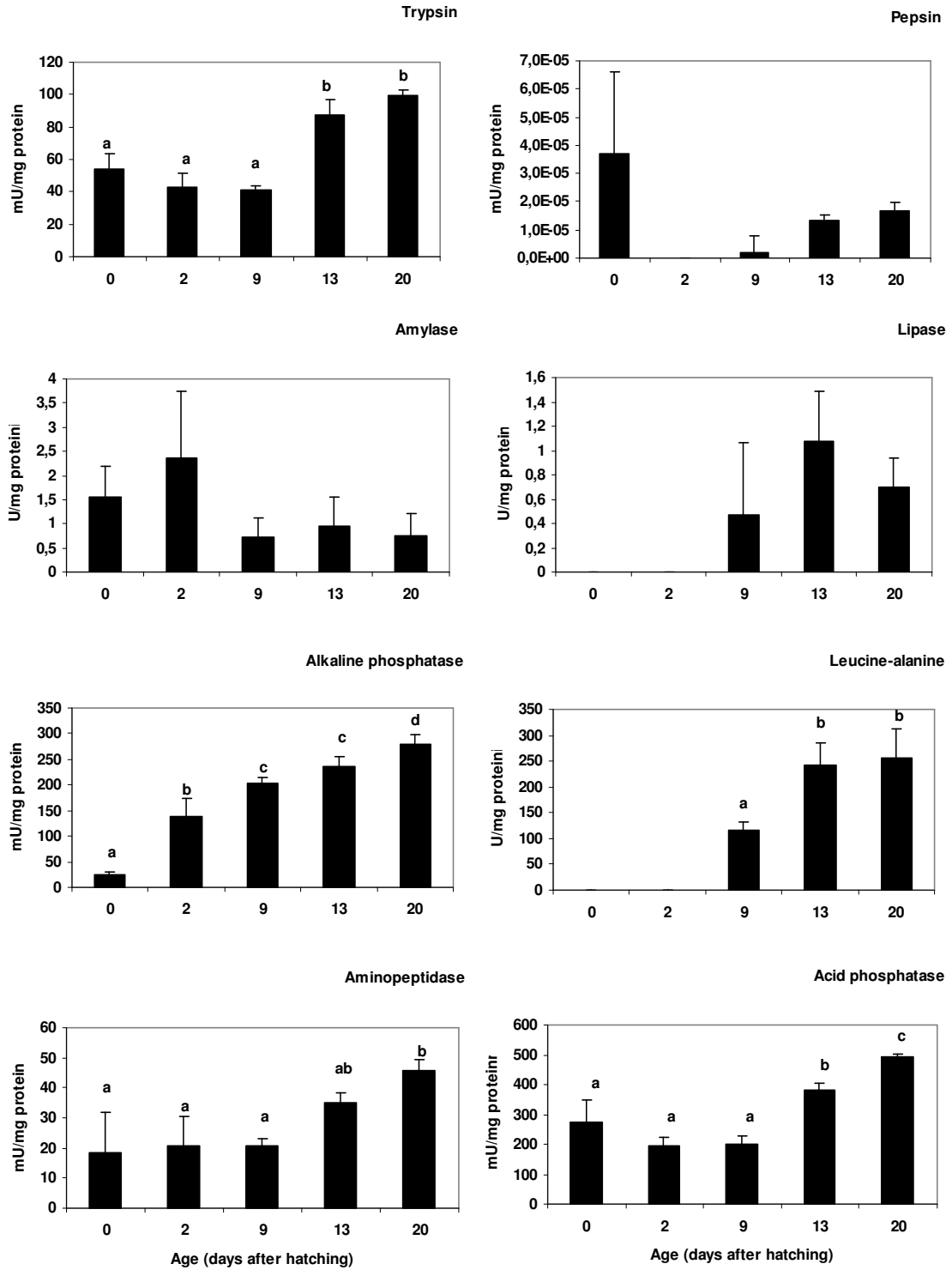

Fig. 12 – Specific activity of digestive enzymes during *Diplodus sargus* larvae development. Enzymes were assayed in whole larvae. Results are expressed as means ± SD (n=3). Different letters indicate significant differences between means.

Trypsin and amylase total activity (Fig. 13) presented the same pattern observed for specific activity, increasing significantly until 20 DAH. Amylase total activity of fish larvae aged 0 and 2 DAH was statistically different from amylase activity for fish larvae at 20 DAH, being 6 and 4.5 times higher, respectively.

Pepsin total activity (Fig. 13) was not detected on fish larvae aged 2 DAH, although after this age pepsin total activity increased, to attain its highest level on fish larvae aged 20 DAH ($P<0.05$).

Lipase total activity (Fig. 13) was only detected at 9 DAH and the activity increased until 20 DAH being at this age significantly higher than at 9 DAH.

Alkaline phosphatase, aminopeptidase and acid phosphatase total activities (Fig. 13), increased until 20 DAH, having the slower increase between 0 and 2 DAH. Alkaline phosphatase total activity at 20 DAH was about 3.2 times higher than at 9 and 13 DAH and about 57 times higher than at 0 and 2 DAH. Fish larvae exhibited significantly different aminopeptidase and acid phosphatase total activities during the first 20 days.

Leucine-alanine total activity (Fig. 13) was only detected at 9 DAH, and increased steadily until 20 DAH.

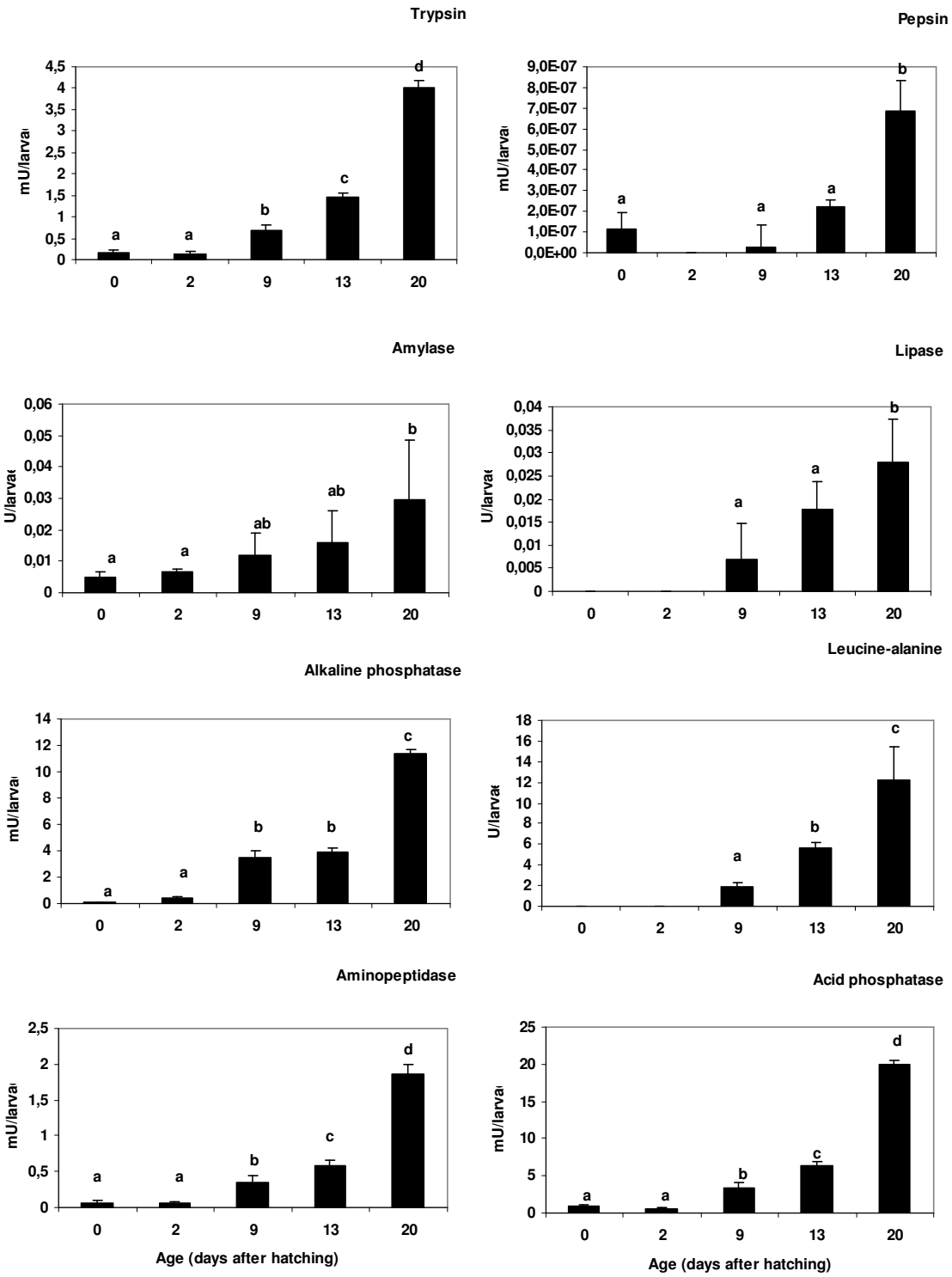

Fig. 13 – Total activity of digestive enzymes during *Diplodus sargus* larvae development. Enzymes were assayed in whole larvae. Results are expressed as means ± SD (n=3). Different letters indicate significant differences between means.

Digestive enzymes activities at weaning

Trypsin specific activity (Fig. 14) of fish larvae from W20 treatment decreased strongly one week after inert diet introduction, although a slight increase was observed on the third week. An opposite pattern was observed for fish larvae from W27

treatment since a slight increase was observed one week after diet introduction, and that level of activity was kept until the end of the third week. At the moment of inert diet introduction (week 0) fish larvae from W20 treatment exhibited a trypsin specific activity 2.3 times superior to fish larvae from W27 treatment, whereas after one week being fed with the inert diet this situation was changed with fish larvae from W20 treatment exhibiting 1.6 times less trypsin activity then fish larvae from W27 treatment.

Amylase specific activity (Fig. 14) of fish larvae from W20 treatment presented a slight decrease one week after diet introduction, being followed by a strong increase on the third week. For fish larvae from W27 treatment amylase specific activity increased until the end of the experiment. One week after the inert diet introduction fish larvae from W27 treatment present amylase activity 2.4 times higher than fish larvae from W20 treatment.

Pepsin specific activity (Fig. 14), of fish larvae from W20 treatment decreased slightly during the first week to increase strongly on the last week of experiment. Fish larvae from W27 treatment exhibited an increase on pepsin specific activity until the end of the experiment. When inert diet was introduced fish larvae from W20 treatment presented significantly higher level of pepsin activity than larvae from W27 treatment, however 1 week after diet introduction this situation was changed with fish larvae from W27 treatment presenting significantly higher levels of activity than larvae from W20 treatment.

Lipase specific activity (Fig. 14), in both treatments, increased until the end of the experiment. One week after inert diet introduction fish larvae from W27 treatment exhibited 2.2 times more activity than fish larvae from W20 treatment.

Alkaline phosphatase and aminopeptidase specific activities (Fig. 14), of white seabream larvae exhibited a similar pattern after inert diet introduction. For both enzymes specific activity of larvae from W27 treatment increased one week after inert diet introduction and decreased on the third week, while the activity on larvae from W20 treatment kept relatively constant during the experiment. Both enzymes were significantly different on week zero with the activity on larvae from W20 treatment being higher than in larvae from W27 treatment.

Leucine-alanine specific activity (Fig. 14), in larvae from W20 treatment decreased slightly on week one and kept the level of activity until the end of the experiment, in treatment W27 the activity decreased until the end of the experiment.

Acid phosphatase specific activity (Fig. 14), in larvae from W20 treatment, had a sudden decrease in the first week and kept more or less in the same level until the end of the experiment. In larvae from treatment W27 the level of activity was kept more or

less in the same level until the end of the experiment. At point 0 fish larvae from W20 treatment exhibited 2.2 times more activity than fish larvae from W27 treatment.

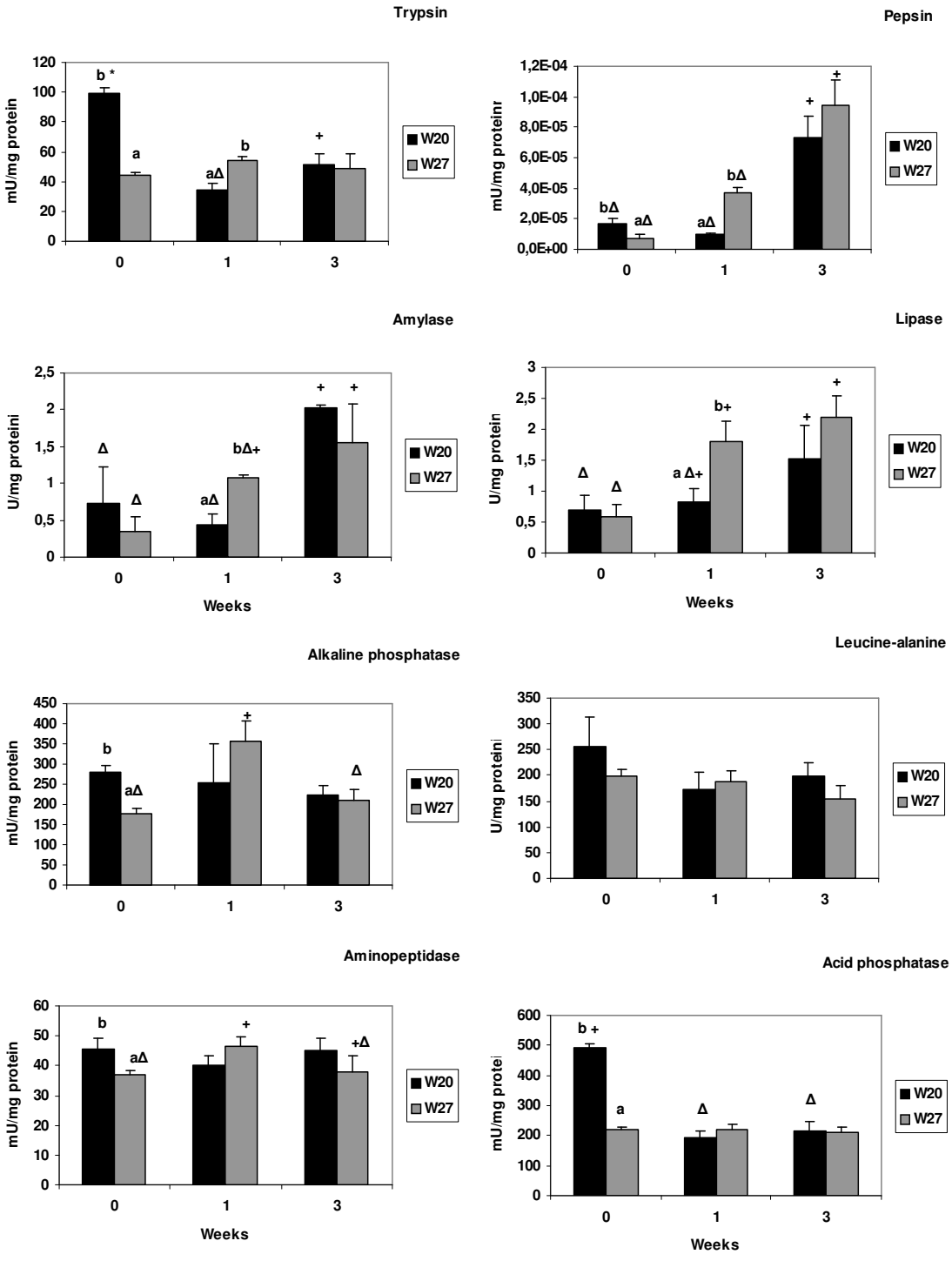

Fig.14 - Specific activity of digestive enzymes during *Diplodus sargus* larvae development. Enzymes were assayed in the dissected abdominal cavity. Results are expressed as means ± SD (n=3). Different letters indicate significant differences between treatments and different symbols indicate significant differences between different stages of larval development within each treatment.

Alkaline phosphatase and aminopeptidase specific activity determined on the brush border (Fig. 15) had a similar pattern of activity with the activity of larvae from W20 treatment increasing and treatment W27 with an opposite pattern decreased the activity. At the third week after the introduction of the inert diet fish larvae from treatment W20 exhibited a significant higher level of aminopeptidase activity compared with fish larvae from W27 treatment.

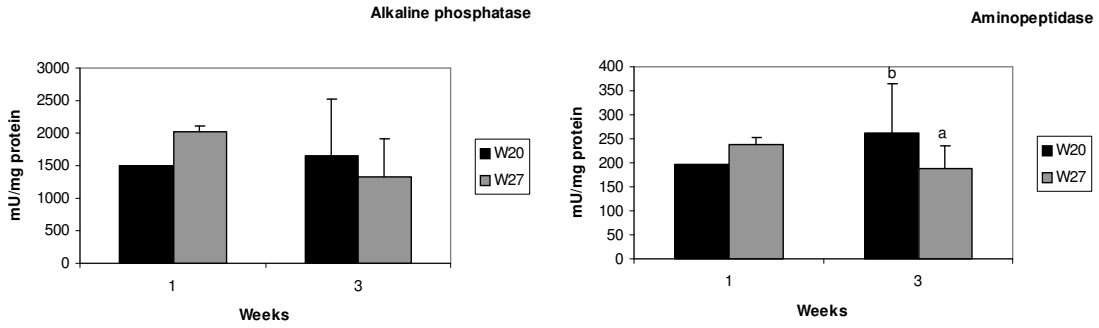

Fig.15 – Specific activity of digestive enzymes during *Diplodus sargus* larvae development. Enzymes were assayed in the brush border segment. Results are expressed as means ± SD (n=3). Different letters indicate significant differences between treatments and different symbols indicate significant differences between different stages of larval development within each treatment.

All the analysed enzymes total activity increased after inert diet introduction (Fig. 16), except for W20 treatment where larvae maintained a similar trypsin and acid phosphatase activity one week after weaning. During the experimental period, fish larvae from W27 treatment exhibited higher levels of enzymes activity when compared with W20 treatment fish larvae, except on week 0 for amylase, pepsin and leucine-alanine.

The differences observed between fish larvae from W20 and W27 treatments were expected since fish started weaning at different stages of development, and bigger larvae usually present higher levels of total activity. However when calculating the relative increase of digestive enzymes for each treatment (Table 7) the results supported the ones obtained with enzymatic total activities since fish larvae from W27 treatment exhibited at one and third week of cultivation a higher relative increase for all analysed enzymes comparing with larvae from W20 treatment.

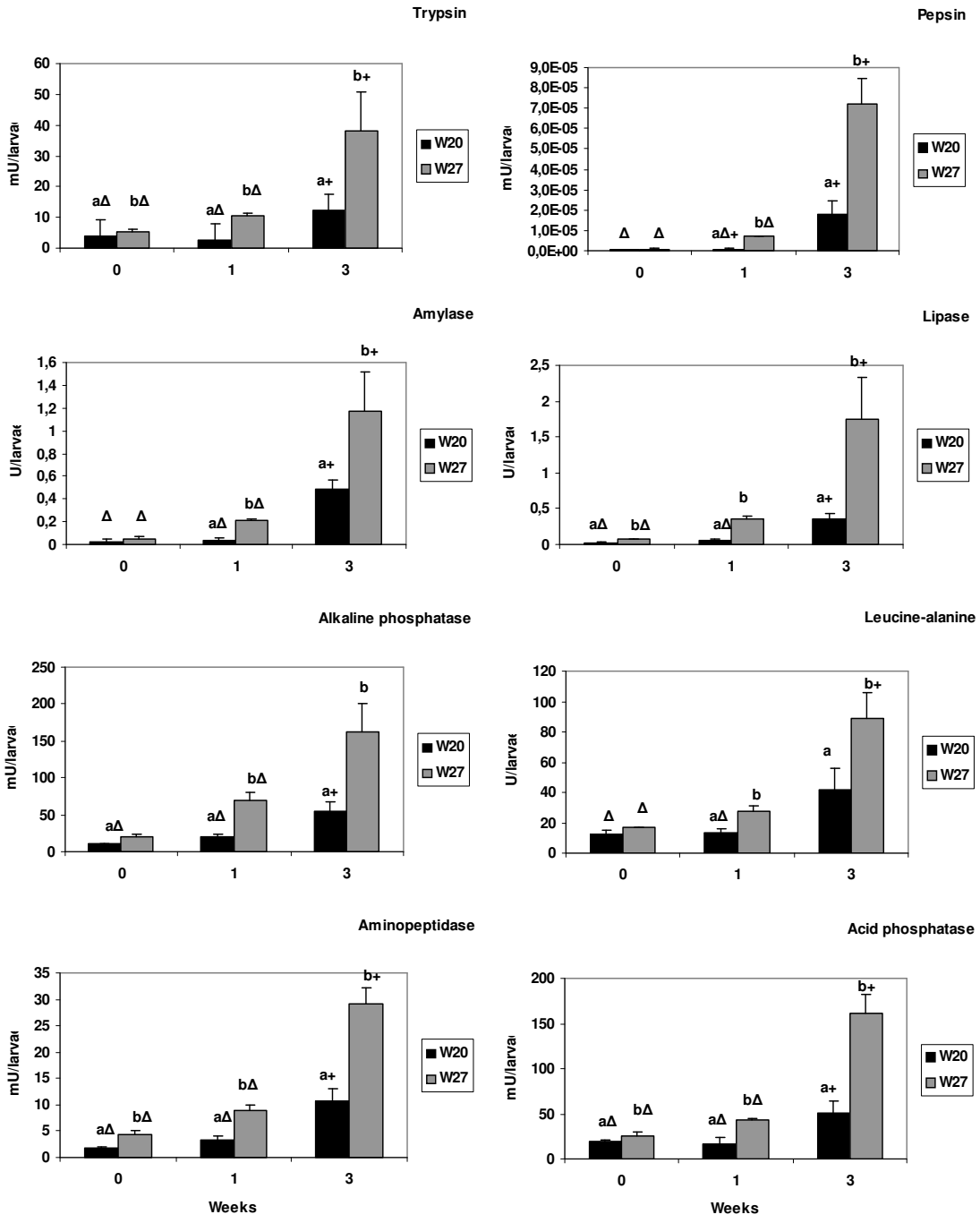

Fig. 16 - Total activity of digestive enzymes during *Diplodus sargus* larvae development. Enzymes were assayed in the dissected abdominal cavity. Results are expressed as means ± SD (n=3). Different letters indicate significant differences between treatments and different symbols indicate significant differences between different stages of larval development within each treatment.

Table 7 – Relative increase activity of total activity of digestive enzymes during *Diplodus sargus* larvae development. Enzymes were assayed in the dissected abdominal cavity.

		Treatments	
Relative increase activity	**Weeks**	**W20**	**W27**
Total activity (mU/larva)			
Trypsin	1	0,693	2,023
	3	3,085	7,332
Amylase (U)	1	1,287	4,865
	3	16,305	27,253
Pepsin	1	1,153	8,215
	3	26,022	81,999
Lipase (U)	1	2,347	5,283
	3	12,475	26,146
Alkaline phosphatase	1	1,745	3,353
	3	4,760	7,835
Aminopeptidase	1	1,775	2,081
	3	5,823	6,667
Leucine-alanine (U)	1	1,119	1,650
	3	3,417	5,384
Acid phosphatase	1	0,819	1,664
	3	2,559	6,218

Alkaline phosphatase and aminopeptidase total activity determined on the brush border (Fig. 17) increased significantly for both treatments until the end of the experiment. Larvae from treatment W27 exhibited a significantly higher activity compared to larvae from treatment W20 for both enzymes during the experimental period.

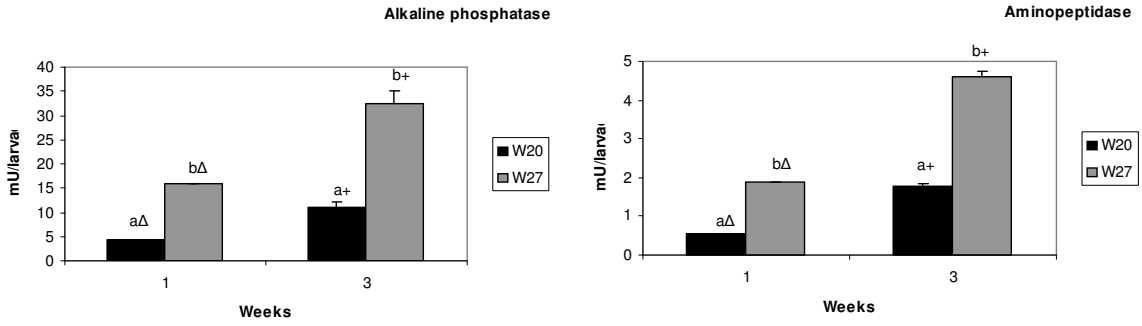

Fig. 17 – Total activity of digestive enzymes during *Diplodus sargus* larvae development. Enzymes were assayed in the brush border segment. Results are expressed as means ± SD (n=3). Different letters indicate significant differences between treatments and different symbols indicate significant differences between different stages of larval development within each treatment.

The enterocyte maturation index (Fig. 18) presented the same pattern of variation both for alkaline phosphatase and aminopeptidase. On week after diet introduction larvae from treatment W27 had higher enterocyte maturation, but on the

third week after the introduction of the inert diet larvae from both treatments had a similar percentage of enterocyte maturation.

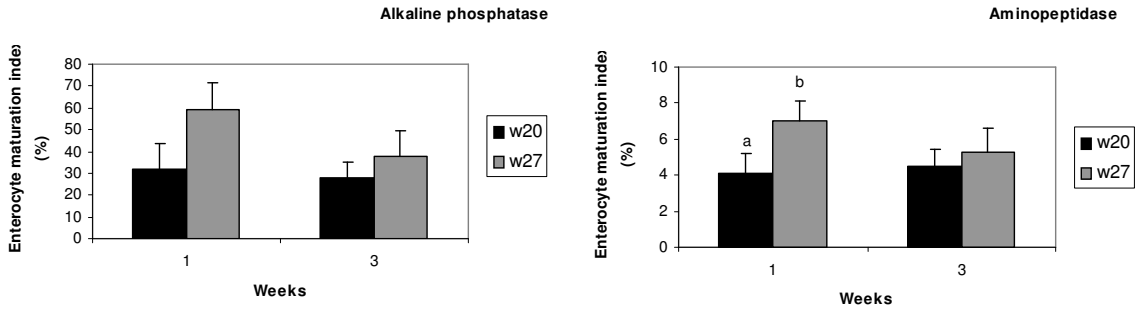

Fig. 18 – Enterocyte maturation index (%) determined using the total activity of brush border enzymes and leucine-alanine peptidase, 1 and 3 weeks after the introduction of the inert diet in *Diplodus sargus* feeding regime. Results are expressed as means ± SD (n=3). Different letters indicate significant differences between treatments and different symbols indicate significant differences between different stages of larval development within each treatment.

S. senegalensis

Enzymatic activity in sole post larvae digestive tract

Trypsin specific activity (Fig. 19) for fish post larvae from both treatments decreased significantly until 40 DAH, having a similar level of activity.

Amylase specific activity (Fig. 19) for post larvae from SW treatment was slightly higher, although without significant differences, than post larvae from CF treatment. Both treatments kept a relatively constant level of activity until the end of the experimental period.

Pepsin specific activity (Fig. 19) for post larvae from SW treatment increased, not significantly, until the end of the experimental period, while the activity on post larvae from CF treatment increased slightly at 35 DAH, but there was a high variability in enzymatic activity in both treatments.

Both treatments presented a similar level of alkaline phosphatase specific activity (Fig. 19) at all ages, decreasing significantly ($P<0.05$) at 40 DAH.

Sole post larvae aminopeptidase (Fig. 19) presented a similar level of activity for both treatments. Post larvae aminopeptidase activity increased slightly at 35 DAH but at 40 DAH it returned to the anterior level of activity.

Leucine-alanine specific activity (Fig. 19) for post larvae from SW treatment was constant in all ages, while post larvae from CF treatment had a significantly higher level

of activity at 35 DAH. In fact at this age post larvae from CF treatment exhibited 1.8 times higher activity than post larvae from SW treatment.

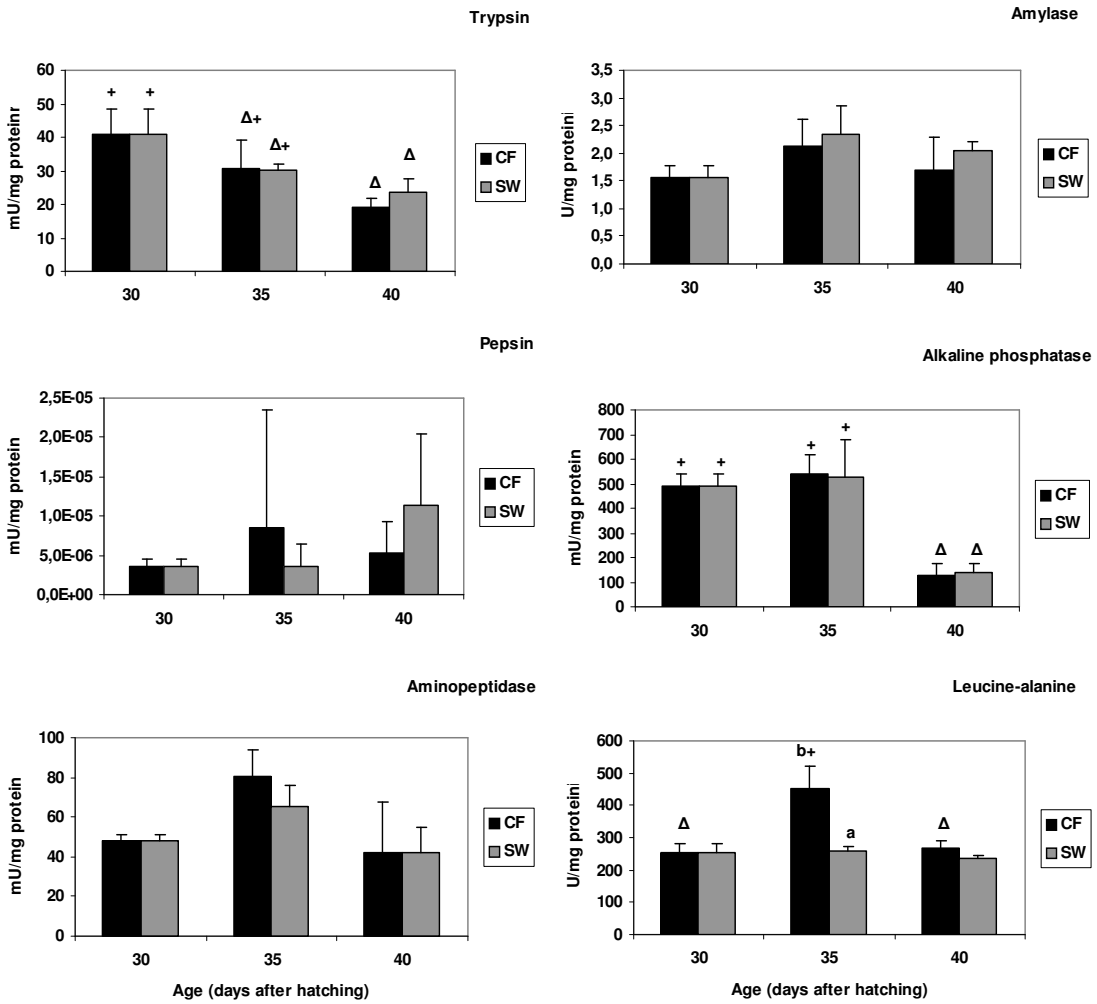

Fig. 19 - Specific activity of digestive enzymes during *Solea senegalensis* post larvae development. Enzymes were assayed in the dissected abdominal cavity. Results are expressed as means ± SD (n=3). Different letters indicate significant differences between treatments and different symbols indicate significant differences between different stages of larval development within each treatment.

Alkaline phosphatase specific activity, determined in post larvae intestinal brush border (Fig. 20), decreased significantly at 40 DAH, for both treatments. Aminopeptidase specific activity, determined in post larvae intestinal brush border in treatment SW decreased significantly at 40 DAH while post larvae from CF treatment kept a level of activity more or less constant until 40 DAH.

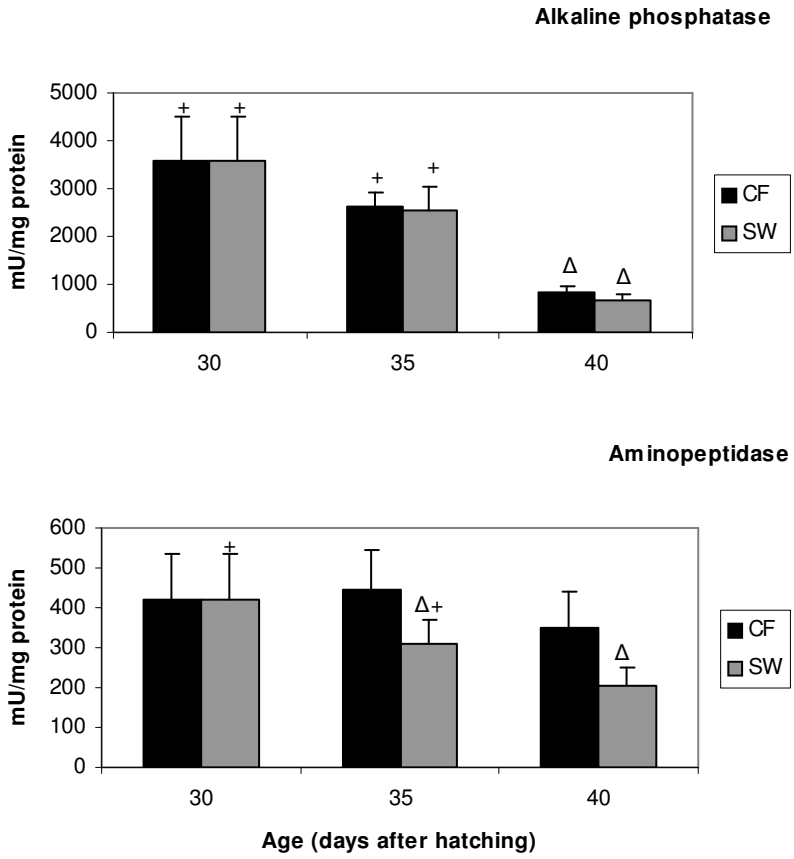

Fig. 20 – Specific activities of alkaline phosphatase and aminopeptidase, of *Solea senegalensis* post larvae, determined in the brush border segment. Results are expressed as means ± SD (n=3). Different letters indicate significant differences between treatments and different symbols indicate significant differences between different stages of larval development within each treatment.

Trypsin, amylase, pepsin and alkaline phosphatase total activity (Fig. 21) exhibited a pattern similar of specific activity. For these enzymes post larvae from treatment SW had a slightly higher level of activity at 40 DAH, but not significant differences between treatments were observed at any studied age.

Aminopeptidase total activity (Fig. 21) in post larvae from both treatments decreased significantly until 40 DAH. Post larvae aminopeptidase total activity at 35 DAH in CF treatment was significantly higher than the activity in post larvae from SW treatment.

Leucine-alanine total activity (Fig. 21) in post larvae from SW treatment decreased at 40 DAH, while post larvae from CF treatment increased significantly at 35 DAH but at 40 DAH returned to the initial level of activity. At 35 DAH the activity in post larvae from CF treatment is 1.8 times higher than in post larvae from SW treatment.

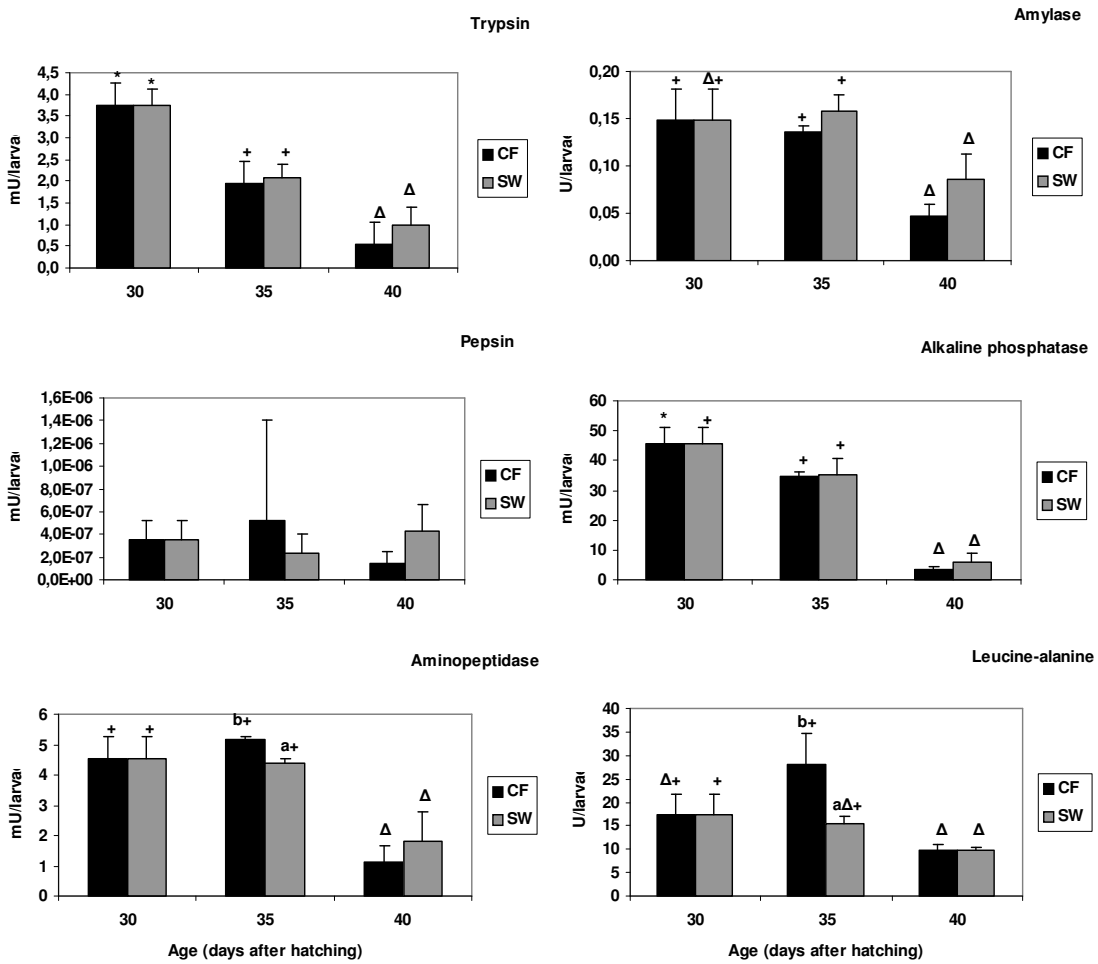

Fig. 21 - Total activity of digestive enzymes during *Solea senegalensis* post larvae development. Enzymes were assayed in the dissected abdominal cavity. Results are expressed as means ± SD (n=3). Different letters indicate significant differences between treatments and different symbols indicate significant differences between different stages of larval development within each treatment.

Alkaline phosphatase total activity determined on the intestinal brush border (Fig. 22) had the same level of activity in post larvae from both treatments decreasing significantly at 40 DAH.

Aminopeptidase total activity determined on the intestinal brush border (Fig. 22) in post larvae from CF treatment increased significantly at 35 DAH, but at 40 DAH returned to the initial level of activity, while larvae from SW treatment presented a relatively constant activity.

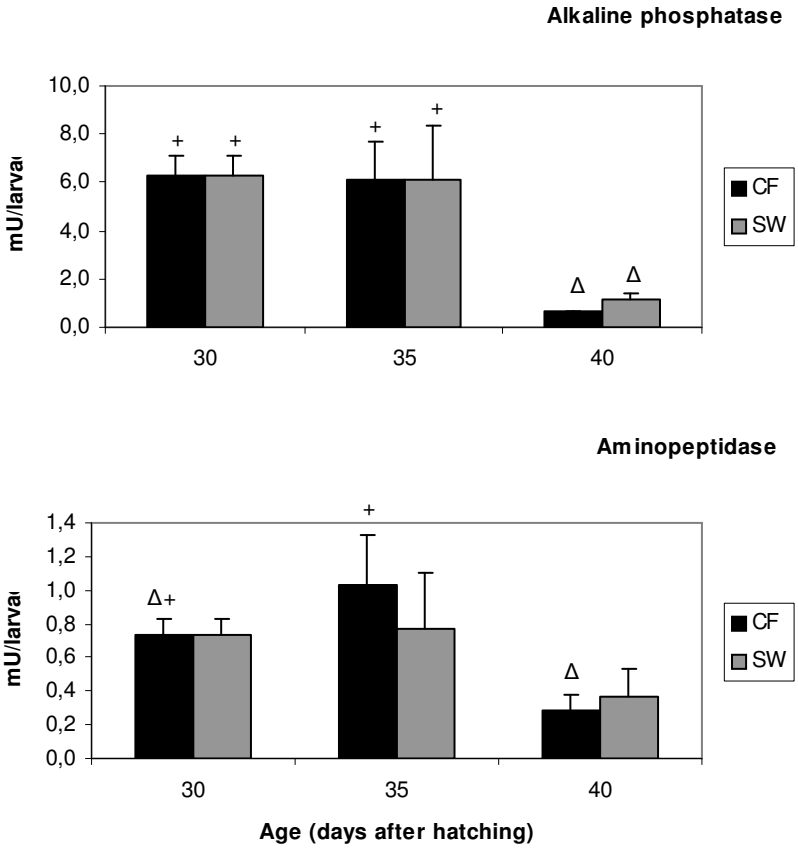

Fig. 22 – Total activities of alkaline phosphatase and aminopeptidase, of *Solea senegalensis* post larvae, determined in the brush border segment. Results are expressed as means ± SD (n=3). Different letters indicate significant differences between treatments and different symbols indicate significant differences between different stages of larval development within each treatment.

Enterocyte maturation index using alkaline phosphatase (Fig. 23) decreased significantly for both treatments until 40 DAH. Post larvae from SW treatment at 40 DAH had a significantly higher level of enterocyte maturation than post larvae from CF treatment. Using aminopeptidase to determine enterocyte maturation index (Fig. 23) there were not differences between treatments or in age, but it can be seen a slightly decrease at 40 DAH.

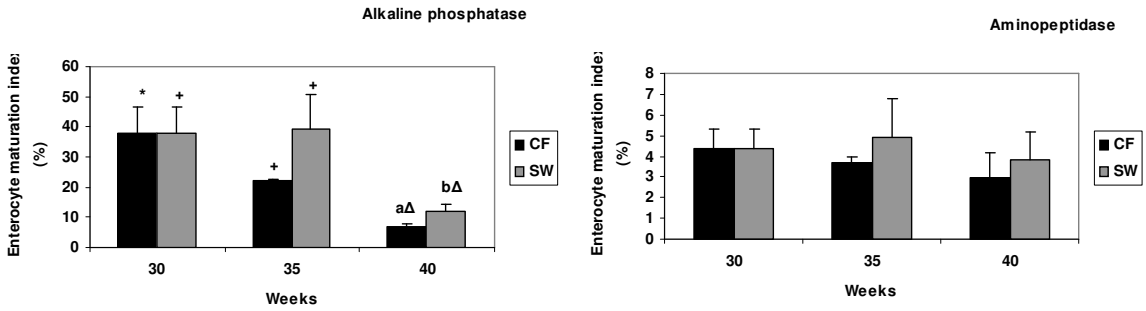

Fig. 23 – Enterocyte maturation index (%) determined using the total activity of brush border enzymes and leucine-alanine peptidase of *Solea senegalensis* post larvae. Results are expressed as means ± SD (n=3). Different letters indicate significant differences between treatments and different symbols indicate significant differences between different stages of larval development within each treatment.

Enzymatic activity on pancreatic and intestinal segment after dissection

Trypsin specific activity (Table 8) determined in pancreas at 50 DAH was 2.5 times higher in post larvae from CF treatment compared with post larvae from SW treatment.

Amylase specific activity (Table 8) determined in intestine from post larvae of CF treatment increased significantly the activity in the end of the experiment (60 DAH).

Alkaline phosphatase specific activity (Table 8) in post larvae intestinal brush border (CF treatment) significantly increased the activity at 60 DAH, whereas in SW treatment post larvae intestine decreased the activity at 60 DAH.

Aminopeptidase specific activity (Table 8) in post larvae from CF treatment significantly increased at 60 DAH in both intestine and intestinal brush border, compared with 50 DAH.

Table 8 – Specific activities of pancreatic and intestinal enzymes, of *Solea senegalensis* post larvae. Results are expressed as means ± SD (n=3). Different letters indicate significant differences between treatments and different symbols indicate significant differences between different stages of larval development within each treatment.

		Treatments	
Digestive enzyme activity	**Days**	**CF**	**SW**
Specific activity (mU/mg protein)			
Trypsin			
Pancreas	50	$23,7^{b}$ ± 8,03	$9,6^{a}$ ± 6,62
	60	11,2 ± 4,89	13,72 ± 13,34
Intestine	50	33,89 ± 14,93	39,64 ± 12,67
	60	20,54 ± 5,68	19,44 ± 2,59
Amylase (U)			
Pancreas	50	0,84 ± 0,06	0,85 ± 0,41
	60	0,72 ± 0,20	0,81 ± 0,03
Intestine	50	$1,32^{\Delta}$ ± 0,36	1,81 ± 0,55
	60	$2,82^{+}$ ± 0,20	2,09 ± 1,51
Pepsin			
Pancreas	50	6,31* ± 1,50*	2,39* ± 4,75*
	60	5,70* ± 5,47*	3,71* ± 2,67*
Alkaline phosphatase			
Brush border	50	$1419,56^{\Delta}$ ± 563,06	1558,80 ± 260,25
	60	$7586,30^{+}$ ± 1503,11	4152,71 ± 2154,27
Intestine	50	433,19 ± 1,05	$567,06^{+}$ ± 2,31
	60	535,29 ± 142,79	$283,45^{\Delta}$ ± 11,71
Aminopeptidase			
Brush border	50	$169,94^{\Delta}$ ± 61,14	187,34 ± 74,83
	60	$1139,65^{+}$ ± 318,74	826,25 ± 460,06
Intestine	50	$31,32^{\Delta}$ ± 0,64	46,31 ± 14,13
	60	$65,35^{+}$ ± 20,33	45,05 ± 1,67
Leucine-alanine (U)			
Intestine	50	298,67 ± 62,43	340,77 ± 35,05
	60	408,98 ± 159,57	286,08 ± 15,85

* Values are multiplied by 10^{-6}.

The values of total activity remained almost unchanged between the two ages analysed and treatments (Table 9). The exception was trypsin analysed in post larvae pancreas that at 50 DAH had 3.4 times higher activity in post larvae from CF treatment compared to post larvae from SW treatment. For both pancreas and intestine post larvae digestive enzymes total activity decreased significantly at 60 DAH.

Table 9 – Total activities of pancreatic and intestinal enzymes, of *Solea senegalensis* post larvae. Results are expressed as means ± SD (n=3). Different letters indicate significant differences between treatments and different symbols indicate significant differences between different stages of larval development within each treatment.

		Treatments	
Digestive enzyme activity	**Days**	**CF**	**SW**
Total activity (mU/larva)			
Trypsin			
Pancreas	50	$1,66^{b+}$ ± 0,44	$0,49^{a}$ ± 0,30
	60	$0,56^{\Delta}$ ± 0,19	0,77 ± 0,71
Intestine	50	$2,19^{+}$ ± 0,86	1,92 ± 1,32
	60	$0,64^{\Delta}$ ± 0,24	1,00 ± 0,30
Amylase (U)			
Pancreas	50	0,06 ± 0,004	0,05 ± 0,03
	60	0,02 ± 0,02	0,05 ± 0,003
Intestine	50	0,086 ± 0,022	0,08 ± 0,027
	60	0,061 ± 0,017	0,09 ± 0,034
Pepsin			
Pancreas	50	4,54* ± 1,11*	1,93* ± 2,79*
	60	2,89* ± 2,61*	2,12* ± 1,35*
Alkaline phosphatase			
Brush border	50	5,17 ± 1,05	4,16 ± 0,45
	60	3,71 ± 0,44	2,92 ± 1,85
Intestine	50	27,66 ± 5,91	24,96 ± 7,39
	60	15,83 ± 1,01	15,16 ± 6,92
Aminopeptidase			
Brush border	50	0,62 ± 0,10	0,50 ± 0,17
	60	0,56 ± 0,12	0,57 ± 0,34
Intestine	50	2,07 ± 0,32	2,18 ± 1,28
	60	1,93 ± 0,23	2,41 ± 1,09
Leucine-alanine (U)			
Intestine	50	11,23 ± 3,24	11,82 ± 1,43
	60	15,09 ± 5,99	12,36 ± 0,43

***** Values are multiplied by 10^{-7}.

Trypsin secretion (Fig. 24) at 50 DAH in fish from SW treatment was 1.4 times higher than trypsin secretion for fish from CF treatment. Trypsin secretion in fish from SW treatment decreased significantly at 60 DAH.

There were not significant differences concerning amylase secretion (Fig. 24) neither between treatments nor between different stages of larval development within each treatment.

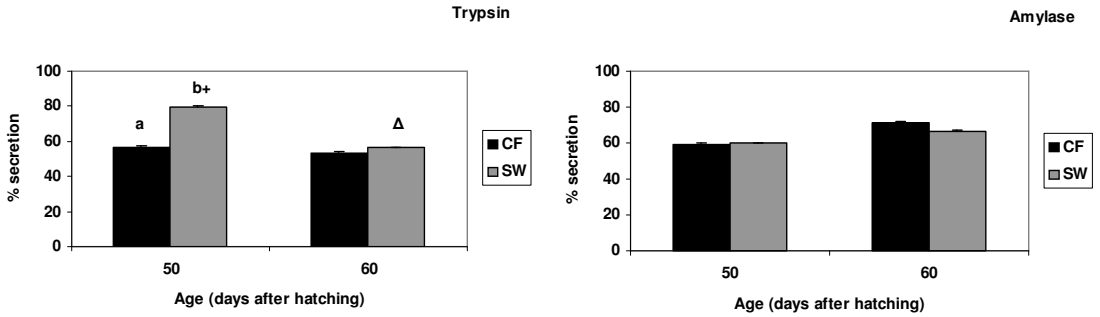

Fig. 24 – Trypsin and amylase secretion level, of *Solea senegalensis* post larvae. Results are expressed as means ± SD (n=3). Different letters indicate significant differences between treatments and different symbols indicate significant differences between different stages of larval development within each treatment.

Enterocyte maturation index using aminopeptidase (Table 10) had no significant differences.

Enterocyte maturation index using alkaline phosphatase (Table 10) in post larvae from CF treatment at 50 DAH had a significantly higher activity compared with post larvae from SW treatment. The level of activity decreased in post larvae from CF treatment from day 50 to 60 DAH.

Table 10 - Enterocyte maturation index (%) determined using the activity of the brush border enzymes and leucine-alanine at 50 and 60 DAH on *Solea senegalensis* post larvae. Results are expressed as means ± SD (n=3). Different letters indicate significant differences between treatments and different symbols indicate significant differences between different stages of larval development within each treatment.

		Treatments	
Enterocyte maturation index (%)	**Days**	**CF**	**SW**
Alkaline phosphatase	50	$46,77^{b+} \pm 3,83$	$35,32^{a} \pm 3,08$
	60	$28,00^{\Delta} \pm 9,39$	23,40 ± 14,17
Aminopeptidase	50	5,66 ± 0,66	4,13 ± 0,99
	60	4,17 ± 0,85	4,54 ± 2,58

4. Discussion

D. sargus

Growth and ontogeny of digestive enzymes

During the studied period white seabream larval growth was identical to the growth reported for this species in previous studies (Saavedra *et al.,* 2006). White seabream larvae showed an exponential growth similar with the ones obtained for other species such as *Pagellus bogaraveo, Diplodus puntazzo, Sciaenops ocellatus* and *Dicentrarchus labrax* (Zambonino-Infante and Cahu, 1994; Lazo *et al.*, 2007; Suzer *et al.*, 2007; Ribeiro *et al.*, 2008).

Cara *et al.* (2003) observed that from 3 to 20 DAH white seabream larvae increased their weight 21 times, whereas in this experiment at 20 DAH larvae had increased their weight only 16 times.

The values of digestive enzymes specific activity for white seabream presented some fluctuation for the first ages, but tended to stabilize around 20 DAH. The general pattern obtained for some digestive enzymes agrees with the ones obtained for *Diplodus puntazzo*, *Pagellus bogaraveo* and *Diplodus sargus* (Cara, *et al.*, 2003; Suzer *et al.*, 2007; Ribeiro *et al.*, 2008).

The majority of digestive enzymes exhibited activity before first feeding, as observed for other fish species (Ribeiro *et al.*, 1999a; Lazo *et al.*, 2007), indicating that enzyme production was not induced by food but must be controlled by genetic mechanisms.

Trypsin is specialized in degrading proteins (Zambonino-Infante and Cahu, 2007) and in this experiment the specific activity for white seabream had a different pattern of activity when compared with the values reported for other species. In other species, such as *Sciaenops ocellatus* and *Solea senegalensis* trypsin activity decreased with age, reaching the lowest values around 20 DAH (Ribeiro *et al.*, 1999b; Lazo *et al.*, 2007). However *Dicentrarchus labrax* and *Diplodus puntazzo* have their peak of activity around 20 DAH, similar with the one obtained in this study (Zambonino-Infante and Cahu, 1994; Suzer *et al.*, 2007). Those differences between species can be due to different development rates that are related with water rearing temperature, since warmer temperatures lead to activity peaks at early stages, whereas colder temperatures lead to activity peaks at later stages of development (Ribeiro *et al.*, 2008). The differences found in trypsin activity between species might be related with the biology of each species and the specific protein requirements. The introduction of *Artemia* on the fish larvae feeding regime might explain an increase on the specific

activity of trypsin, as it was observed for common pandora, or affect the level of activity if introduced late in the diet as it was observed with blackspot seabream (Suzer *et al.*, 2006; Ribeiro *et al.*, 2008). In this study the increase at 13 DAH can not be explained by the introduction of *Artemia sp* in the diet, because this food item was only introduced at 13 DAH, and the sampling for enzymatic analysis was done before the morning meal. Differences in trypsin activity can be explained by different abiotical factors such as illumination, salinity and pH (Suzer *et al.*, 2007).

At 2 DAH larvae exhibited a peak of amylase specific activity that decreased after this age. This means that larvae of white seabream have the ability to use carbohydrates since mouth opening, as it was observed for other marine fish species (Ribeiro *et al.*, 1999a; Cara, *et al.*, 2003; Lazo *et al.*, 2007; Suzer *et al.*, 2007; Ribeiro *et al.*, 2008). It was suggested that the decrease in amylase activity few days after hatching may be genetically programmed and that amylase fluctuations may be independent of the dietary glucide concentration (Cahu and Zambonino-Infante, 2001).

Lipase is one of the main lipolitic enzymes and is responsible for the catalysis of carboxy-ester bonds (Cahu and Zambonino-Infante, 2001; Zambonino-Infante and Cahu, 2007). In this study lipase specific activity decreased after 13 DAH which could be due to changes in the diet, since at 13 DAH *Artemia* nauplii were introduced in the feeding plan of fish larvae. *Artemia sp* and rotifers were enriched with different products, Rich Advanced® and Protein Selco® respectively, which according to manufacture specifications presents a different fatty acid composition, besides the specific differences on fatty acid content of live prey species. The different enrichment products might have influenced the lipid values. Larvae need time to adapt to new kinds of feed, reason why the enzymatic activity changes (Kolkovski, 2001). Changes in enzymatic activity can be a result of an adaptation to changes in the composition of live feed or a result of growth and development of the tissues and organs (Martinez *et al.*, 1999). It was reported for *S. senegalensis* a maximal lipase activity at 10 DAH which were probably related with the development of exocrine pancreas (Martinez *et al.*, 1999), which could be the explanation for the high activity detected at 13 DAH in white seabream larvae. Lipase activity was not affected by dietary lipid level on *Pagellus bogaraveo* larvae, but was affected by fatty acid component (Ribeiro *et al.*, 2008).

Pepsin is one of the enzymes responsible for protein digestion, although it is usually detected at later stages of larvae development, since in the first days after hatching fish larvae does not have a functional stomach (Zambonino-Infante and Cahu, 2001). In this work pepsin presented the higher level of activity at hatching. The reason for this might be related to the proteases involved in the process of hatching influencing

the value of pepsin. There are lysosomal chatepsins present in the larval body that can be mistaken as pepsin, when using whole larval body to determine enzymatic activity (Lazo *et al.*, 2007). Lazo *et al.* (2007) reported that recently several authors reported pepsin activity before gastric glands maturation, when the methodology applied to determine pepsin activity used haemoglobin as substrate. Nevertheless, it seems not to be the case, since pepsin activity for Senegalese sole was only detected after the appearance of gastric glands using haemoglobin as substrate (Ribeiro *et al.*, 1999a). Martinez *et al.* (1999) reported pepsin activity from day 9 in *S. senegalensis*, but the same authors more recently justified those results with the presence of cathepsines related to the mobilization of protein in body tissues (Fernandez *et al.*, 2001).

The typical process of intestine maturation is characterized by a progressive increase in the activities of the brush border membrane enzymes and a decrease in the activities of the cytosolic enzymes (Zambonino-Infante and Cahu, 2001). Leucine-alanine and acid phosphatase are responsible for completing protein digestion, function that begun partly to be done by alkaline phosphatase and aminopeptidase when the maturation of the enterocytes took place (Cara *et al.*, 2003).

In this study acid phosphatase had the maximal value of enzymatic activity at 20 DAH, while Cara *et al.* (2003) with the same species at the same age obtained the lowest value of activity. Nevertheless both studies had high activity of acid phosphatase revealing the great importance of pinocytosis in the early life stages of larval development.

Leucine-alanine activity reflects also the importance of the pinocytosis in the early stages of development, maintaining a similar level of activity until 20 DAH. An increase in leucine-alanine activity might reflect an increasing protein digestive ability along the developmental period (Kvåle, 2006).

White seabream exhibited normal values of aminopeptidase and alkaline phosphatase activities during the larval stage. Results obtained in this work were similar with the ones obtained for sea bass in the first days of cultivation with live feed (Zambonino-Infante and Cahu, 1994).

Total activity of digestive enzymes increased with age for all enzymes analysed, indicating that larvae enhanced their digestive capacity during the first stages. The increase in enzymatic activity is due to an increase in the larva size (Zambonino-Infante and Cahu, 2001). This is a general pattern observed for many species such as *Diplodus puntazzo, Solea senegalensis, Pagellus bogaraveo, Sciaenops occellatus* (Suzer *et al.*, 2007; Ribeiro *et al.*, 1999a; Ribeiro *et al.*, 2008; Lazo *et al.*, 2007).

Weaning at different stages of development

Suzer *et al.* (2007) working with *Diplodus puntazzo* until 50 DAH obtained a specific growth rate (SGR) of 5.9 $\%d^{-1}$, whereas in this experiment white seabream exhibited a mean value SGR of 4.2 $\%d^{-1}$ for both treatments (results not showed). Results obtained by Pousão-Ferreira *et al.* (2005) showed that *D. sargus* grows faster than *D. puntazzo*, however in this study only for total length the results were higher than the ones obtained by Suzer *et al* (2007) with *D. puntazzo*.

Although RGR of fish larvae from W20 treatment was affected one week after diet introduction, they exhibited a growth capacity to equal the RGR from fish larvae from W27 treatment, ending with similar RGR. Nevertheless, it must be keep in mind that fish larvae from W20 treatment were 50% lighter than fish larvae from W27 treatment, although this percentage was reduced to 15% if we compare weights at similar ages (weight calculated using growth curve).

Suzer *et al.* (2007) with *D. puntazzo* obtained a survival rate of 21.7 % a relatively higher survival compared with the one obtained in this work (3.3 % for treatment W20 and 3.8 % for treatment W27). With the same species Saavedra *et al*. (2006) obtained better survivals, 18.7 % than in the present work. In this experiment mortality was only determined in the end of the experimental period and not before and after the introduction of inert diet. The low survival obtained was a result of the high mortality recorded before the weaning period, during the weaning period there was not a significant mortality, the high levels of mortality stopped around 19 DAH.

Pancreatic enzymes specific activities of larvae from W20 treatment decreased their activity one week after the introduction of the diet. However in the third week larvae were able to recover their enzymatic activities. It seems that the early introduction of inert diet in white seabream feeding regime affected their enzymatic activity in a negative way. Nevertheless larvae were able to recover from this early weaning reaching the same levels of activity by the end of the experimental period as larvae from W27 treatment. Specific activity of pancreatic enzymes for larvae from W27 treatment tended to increase until the end of the experiment except for trypsin that kept relatively constant until the end of the experiment. The increase in enzymatic activity observed in this study, except for trypsin, might reflect the beginning of acid digestion thus providing more substrates for other digestive enzymes activities and decreasing the importance of trypsin activity (Cara *et al.*, 2003).

The relatively constant level of trypsin activity can be related with a gastric digestion that might already be observed at this stage of fish development as it was observed for *P. bogaraveo* (Ribeiro *et al.*, 2008). Moreover, it has been reported that in other sparids a decline in activity was observed after 20 DAH, as it was observed for

white seabream in this experiment, which was coincident with changes in feed regime and brush border maturation (Suzer *et al.*, 2007). The presence of anti-tryptic factors in diet binders has also been described to affect trypsin activity (Moyano *et al.*, 1999), nevertheless the inert diet given to white seabream larvae had none of these components as ingredient.

The increase in activity of amylase and lipase for fish larvae from both treatments could be due to an adaptation to the composition of the inert diet. In fact, it was reported that *Pagellus bogaraveo* adapted easily to inert diet without additional effort because the level of amylase after the introduction of inert diet was not significantly affected, however the level of lipase activity suffered a drastic increase after inert diet introduction (Ribeiro *et al.*, 2008). In contrast, *Diplodus puntazzo* exhibited a sudden increase in amylase activity coinciding with inert diet introduction, and had also an increase of lipase activity related with triglyceride content of the extruded compound diet (Suzer *et al.*, 2007), a similar pattern might have happened with white seabream in this experiment.

The increase of pepsin activity might be related with the fully stomach maturation, as it was reported the appearance of gastric glands at 20 DAH for *D. sargus* and that at 30 DAH larvae had an almost fully functional stomach (Cara *et al.*, 2003). Between 32 and 40 DAH for *D. Puntazzo* it was reported stomach complete functionality (Suzer *et al.*, 2007).

Intestinal enzymes after the beginning of treatments were only significantly different on the week of the introduction of inert diet meaning that enzymatic activity in white seabream larvae was not affected by the early introduction of inert diet in larvae feeding regime.

Alkaline phosphatase and aminopeptidase had more or less the same level of activity after weaning meaning that the intestine seemed to be mature and that the early introduction of inert diet did not affected the intestinal maturation. Although it was not a significant difference, alkaline phosphatase and aminopeptidase in the third week after the introduction of the inert diet had a slightly higher level of activity for larvae from W20 treatment suggesting a better developmental state compared with larvae from W27 treatment. The unchanged values of alkaline phosphatase at the post-natal stage in *P. bogaraveo* were attributed to an alteration in the absorption pattern by intestine due to an introduction of an inert diet with a different lipid constitution from the live feed that fish larvae were ingesting before the inert diet (Ribeiro *et al.*, 2008). This could be the reason for constant alkaline phosphatase and aminopeptidase level of activity in white seabream larvae.

Aminopeptidase had a significantly higher level of activity on the brush border after three weeks from inert diet introduction on larvae from W20 treatment, reflecting that an early weaning could promote better results in white seabream larvae intestinal maturation.

Acid phosphatase decreased their activity, since with intestine maturation there is a relative decrease of the cytosolic digestion, pinocitosis, due to a concomitant increase of digestion by brush border enzymes (Ribeiro *et al.*, 1999a; Suzer *et al.*, 2007).

In this experiment leucine-alanine peptidase specific activity was kept rather constant as observed for halibut and cod (Kvåle *et al.*, 2007), although an abrupt decreased was observed for sea bass and sole between 20 and 30 DAH (Ribeiro *et al.*, 1999a; Zambonino-Infante and Cahu, 2001). One possible explanation for the constant activity of this enzyme instead of a decrease is a consequence of using different tissues in the homogenate for enzymatic detection. In fact, Kvåle *et al.* (2007) studying Atlantic halibut leucine-alanine peptidase suggested that the lack of decrease in activity could be do to the effect of other tissues present in the intestinal homogenate that masked the changes in ontogenetic leucine-alanine detection.

Three weeks after inert diet introduction for white seabream no significant differences were observed between an early or late introduction of the inert diet meaning that the early weaning did not affect fish larvae digestive capacity. Furthermore, it indicated that white seabream larvae were able to modulate their digestive enzyme activity as it was reported for *Dicentrarchus labrax* weaned at various times after hatching: 10, 15, 20 and 25 days (Cahu and Zambonino-Infante, 1994).

Fish larvae from W27 treatment exhibited higher values of digestive enzymes total activity after the beginning of treatments for all the enzymes analysed, but this higher activity was a consequence of larval size rather than to larval digestive ability. In fact, fish larvae from W27 treatment begun to eat inert diet later, so they were older and more developed than larvae from W20 treatment during all experiment. The bigger size and consequently an increase in enzyme activity per individual were reflected in the higher total activity. During development fish larvae increases their digestive capacity (Zambonino-Infante and Cahu, 2007). When using the relative increase in the total activity it can be excluded the larvae size effect. Comparing the relative increase on enzyme activities after inert diet introduction, the results supported the ones obtained with total activity. Results showed that even excluding the size effect, total enzymatic activities from larvae from W27 treatment growths faster than in larvae from W20 treatment. Thus total activity may reflect a possible higher digestive enzymatic capacity during all experimental period in larvae from W27 treatment compared with

larvae from W20 treatment, but it can be not forget that per mg of protein larvae tended to have the same digestive ability.

Maturation of enterocytes is of great importance for the larvae, since good growth and survival is achieved with early maturation of enterocytes (Cahu and Zambonino-Infante, 2001; Zambonino-Infante and Cahu, 2007). The enterocyte maturation index indicates the level of intestinal maturation, and in this work allowed to observe that it seems advantageous to introduce the inert diet earlier in the feeding regime of white seabream. In the third week after the inert diet introduction larvae from W27 treatment decreased, but not significantly, the percentage of enterocyte maturation suggesting a slightly lower hydrolytic capacity compared with larvae from W20 treatment.

In conclusion:

- Enzymes and level of activity determined in this experiment for white seabream followed a similar pattern of activity observed for other Sparidae species studied to date.
- White seabream had digestive enzymatic activity since hatching reflecting the ability of this species to digest food from early life stages.
- The pattern of digestive enzymes activity was related to organogenesis and the type of food used at different developmental stages.
- Trypsin, amylase, alkaline phosphatase, aminopeptidase and acid phosphatase were detected at hatching, while lipase, pepsin and leucine-alanine were only detected at 9 DAH.
- Digestive capacity was improved at an earlier developmental stage by the contribution of pancreatic enzymes than by brush border enzymes.
- In this study leucine-alanine peptidase could not be used as a marker of larval mode of digestion because the expected decline in activity was not observed.
- The study of digestive enzymatic activity allowed observing that white seabream was prepared to receive inert diet and the effects of weaning at two different ages.
- Data obtained for growth, survival and enzymatic activity after the introduction of the inert diet suggested that white seabream might be weaned from earlier stages since they were able to recover from this early introduction, without major problems.

Further multidisciplinary studies are needed to really understand the pattern of enzymatic activity on white seabream and to optimize larval feeding protocols in order to enhance digestive capacity. It could be studied the possibility and effects of introducing inert diet earlier than 20 DAH in white seabream feeding plan.

S. senegalensis

During the experimental period sole presented an irregular growth, presenting some periods where sole post larvae did not growth for both treatments. Comparing growth, both in weight and length, with other studies for the same species it can be seen that lower growth was obtained for this study. Dinis *et al.* (1999) reported an average total length of around 14.0 mm for 35 DAH larvae, whereas in the present study fish larvae presented an average length of 7.0 mm indicating that fish larvae used in this experiment exhibited a smaller size from the beginning. Ribeiro *et al.* (2002) reported a total length of 13.0 mm and a dry weight of 3.4 mg for sole aged 36 DAH fed with artemia. In this experiment at 35 DAH the mean dry weight of sole of the treatments was 1.96 mg. Cañavate and Fernández-Díaz (1999) obtained with different feeding regimes sole at 60 DAH that varied in dry weight between 15-24 mg, while in this experiment fish with the same age had a mean weight of the treatments of 2.77 mg. Besides the slow growth post larvae from SW treatment had a significantly higher growth than post larvae from CF treatment.

Sole post larvae exhibited a negative RGR for both treatments, as was previously observed by other authors for Sole when studying weaning strategies such as Ribeiro *et al.* (2005) and Engrola *et al.* (2007) with *Solea senegalensis*. Post larvae from SW treatment had the better growth performance and consequently had the higher mean RGR.

In this experiment survival rate varied between 22% and 25%, respectively for CF treatment and SW treatment, which were low survival rates when compared with the values obtained for other works. In fact, Engrola *et al.* (2007) obtained survival rates that varied between 38.46 % and 90.06 % at 47 DAH in an experiment using sudden weaning at different ages. These authors obtained better survivals with sole sudden weaned at 40 DAH (98.76 ± 0.84 %) or with co-feeding at 50 DAH (38.61 ± 3.13 %). The survival rates might be related with tank volume, since Engrola *et al.* (2007) used 4 L tanks whereas in this experiment 300 L tanks were used. So a lower rearing scale than the one used in this experiment.

The low physical condition of larvae in this work was not related with the weaning, since larvae presented a smaller size at the beginning of the study when compared with previous studies for this species. Larvae physical condition can be affected by water conditions, methods of rearing, feeding protocol employed or diet composition (Ribeiro *et al.*, 2008).

The relatively better results obtained with sudden weaning related with co-feeding could be due to a reduced period of co-feeding. In this study 5 days of co-feeding were used although Cañavate and Fernández-Díaz (1999) reported that 7 days could be few

for an acceptable weaning period in sole weaned at 43 DAH. Another reason that could explain the better results obtained with sudden weaning compared with co-feeding was when fish have inert and live feed together they tend to choose only the live feed. In a co-feeding regime the proportion of live feed is reduced until a complete weaning to inert diet, but because larvae choose live feed, they become to eat less when reduces the live feed causing slower growth and mortality by fish that do not eat enough feed to survive. This was proved with *Sparus aurata* that when was fed with live and inert diet together larvae choose preferentially the live feed (Fernández-Díaz *et al*., 1994). Ribeiro *et al.* (2002) with sole noted that larvae had difficulties accepting inert diet, and that the quantity of artemia offered was not enough to satisfy metabolic needs.

Enzymatic activity in sole post larvae digestive tract

Pancreatic enzymes specific activity did not presented significant differences between treatments meaning that the treatments did not affected the activity of these enzymes. Although significant difference were not observed at 40 DAH, larvae from SW treatment presented a higher level of pancreatic enzymes activity suggesting that a sudden weaning may be beneficial for sole post larvae.

Trypsin specific activity in post larvae from both treatments exhibited a significant decrease in activity until 40 DAH, as it was reported for sparids species after 20 DAH, which was related with changes in the feed regime and in brush border maturation (Suzer *et al*., 2007). The presence of a gastric digestion can explain the decrease in trypsin activity, because with the presence of a gastric digestion the digestion of proteins by trypsin might decrease. This is in accordance with data obtained for pepsin activity for fish post larvae from SW treatment that increased the activity at 40 DAH when compared with the activity at 30 DAH. The constant level of trypsin specific activity in *P. bogaraveo* was associated with the presence of a gastric digestion (Ribeiro *et al*., 2008). It was reported for sole fed ICES compound diet the influence of trypsin inhibitors in the activity of trypsin, inhibitors as the ones found in vegetable components or as diet binders (Ribeiro *et al.,* 2002). However in this experiment the decrease in trypsin activity can not be related with the presence of trypsin inhibitors because the inert diet given to sole post larvae had none of these components as ingredient. Trypsin activity might be used as a short time reactive enzyme because the level of activity reacts in the 24h after the changes in feed supply. When the feed supplied is unsuitable for larval needs, larvae tend to increase trypsin production in order to enhance the nutritional yield. The attempt to increase trypsin activity consumes high levels of energy leading to high loss of endogenous proteins, low growth and mortality (Drossou *et al*., 2006).

Amylase specific activity presented a more or less constant level of activity in sole from both treatments suggesting that post larvae adapted easily to the inert diet as it was reported for *Pagellus bogaraveo* which adapted easily to inert diet without additional effort because the level of amylase after the introduction of inert diet was not significantly affected (Ribeiro *et al.*, 2008).

In sole, gastric glands were observed for the first time around 27 DAH (Ribeiro *et al.,* 1999b), so the increase in pepsin specific activity in larvae from both treatments in this experiment might reflect stomach maturation. Ribeiro *et al.* (1999a) did not detected pepsin-like activity until 32 DAH in sole post larvae fed live feed.

Intestinal enzymes specific activity did not presented significant differences between treatments meaning that the treatments did not affected the activity of these enzymes. The exception was for leucine-alanine peptidase that at 35 DAH CF treatment had a significant higher activity, but at 40 DAH these differences disappeared.

Sole alkaline phosphatase specific activity had a significant decrease at 40 DAH, but as a brush border enzyme it was supposed to increase the activity with age, meaning with maturation of intestine. Alkaline phosphatase activity is affected by the amount of ingested diet (Ribeiro *et al.,* 2002), so alkaline phosphatase activity of post larvae at this age could reflect that post larvae were eating less because sole were not adapted yet to the inert diet conditions. Weight and length data support that post larvae were eating insufficiently at this age. Alkaline phosphatase is considered a general marker of nutrient absorption (Cara *et al.*, 2003), reflecting the physical condition of sole post larvae. Engrola *et al.* (2007) suggested that alkaline phosphatase is a good indicator of nutritional status in sole with a weight < 5 mg.

Aminopeptidase is an indicator of complete hydrolysis at an intestinal level (Cara *et al.*, 2003), and in this work it specific activity kept without significant differences until 40 DAH or between treatments. Increase of aminopeptidase on intestinal brush border represents the normal process of enterocyte maturation in fish larvae (Zambonino-Infante and Cahu, 2001) but this was not visible in the present work. The constant level of activity might mean that the intestine seemed to be mature and that besides the type of weaning the inert diet did not affected the maturation process. On the other hand the constant level of activity could show an alteration in the absorption pattern by intestine due to an introduction of a inert diet which as a different composition from the live feed that fish post larvae were ingesting before the inert diet (Ribeiro *et al.*, 2008).

Leucine-alanine peptidase at 35 DAH seems to be less affected by the co-feeding regime, because it has a significantly higher activity, but after this age there are no differences. Leucine-alanine is responsible for the complete hydrolysis at an

intestinal level (Cara *et al.*, 2003) so a co-feeding regime is not an abrupt change to inert diet, allowing the intestine to adapt to the new kind of feed. At 40 DAH the level of activity were similar for co-feed and sudden weaned post larvae which might be related with the fact that at this age larvae from CF treatment were eating only inert diet as larvae from SW treatment.

The pattern seen for alkaline phosphatase on brush border was similar with the one seen in the intestinal homogenate. Aminopeptidase activity in the brush border shown an opposite pattern of the one exhibited on intestine decreasing significantly at 40 DAH in larvae from SW treatment suggesting that a sudden weaning might had adverse effects in the normal process of intestinal renewal.

Enterocyte maturation is of great importance for post larvae, since good growth and survival is achieved with early maturation of enterocytes. This maturation is genetically programmed but an inadequate diet may delay or avoid the enterocyte maturation inducing larvae dead (Cahu and Zambonino-Infante, 2001; Zambonino-Infante and Cahu, 2007). The enterocyte maturation index, using alkaline phosphatase in this work reflected the low physical condition that post larvae had in both treatments at 40 DAH. The activity of this enzyme shows an advantage in introducing the inert diet in a sudden way in the diet of sole, since at 40 DAH post larvae from SW treatment had a significantly higher maturation index than post larvae from CF treatment. Enterocyte maturation index using aminopeptidase did not exhibited significant differences between treatments or between ages which supports the idea that alkaline phosphatase is a better indicator of nutrient absorption, respond better to diet changes (Cahu and Zambonino-Infante, 1994; Cara *et al.*, 2003). The alterations on the enterocyte maturation index using aminopeptidase were less visible which could be due to aminopeptidase might have a conservative role. In fact Cahu and Zambonino-Infante (1994) studying *Dicentrarchus labrax* suffering from severe malnutrition conditions, observed a stable value of aminopeptidase that they suggested to have a conservative role preventing loss of tissue protein in the firsts days. It was reported in cod that aminopeptidase reached a maturational activity level at later stages of development than alkaline phosphatase (Kvåle, 2006), which could happened with white seabream in this experiment.

Total activity increases with age and post larvae size (Zambonino-Infante and Cahu, 2001). In this work the opposite happened, with almost all enzymes total activities decreasing significantly until 40 DAH, except pepsin activity for post larvae from both treatments and aminopeptidase activity from post larvae of SW treatment determined in the brush border. The fact that total activity decreased reflects the low

physical condition presented in post larvae from both treatments, which was supported by weight and length data.

Engrola *et al.* (2007) reported that a sudden weaned regime produced better digestive capacities compared with a co-feeding regime in sole with 40 DAH and 10 mg of dry weight, results in agreement with the ones obtained in the present study.

Enzymatic activity on pancreatic and intestinal segment after dissection

At 50 and 60 DAH enzymatic activities were determined on dissected post larvae, so it was possible to see enzymatic activity in intestine and pancreas separately.

Specific activities of pancreatic enzymes of post larvae from CF treatment tended to decrease between 50 and 60 DAH, except for amylase determined in intestine that was significantly higher at 60 DAH. The higher amylase activity in intestine in post larvae from both treatments may be related with the carbohydrates of the inert diet, this means that there was modulation of the amylase activity by the dietary starch as it happened in sole fed ICES diet (Ribeiro *et al.,* 2002). Although post larvae from SW treatment in some cases behave in the same way as post larvae from CF treatment, there were not significant differences between treatments at the end of the experimental period. The fact that post larvae from both treatments at 60 DAH had a similar level of activity reflected that post larvae were adapted to the inert diet despite the type of weaning. The decrease on enzymatic activity may be associated with the quantity of ingested feed, since post larvae could be eating less by the end of the experimental period. Engrola *et al.* (2007) related the decrease in trypsin activity in sole larvae with the fact that larvae were not eating properly. Fish larvae respond quickly to diet change, since according to Cara *et al.* (2003) fish larvae were able to modulate their trypsin and amylase activity, respectively, in 24h and less than 3 days to a diet alteration.

The type of weaning did not affected secretion by pancreas because there were no differences between treatments, except at 50 DAH for post larvae trypsin activity, but at 60 DAH the difference disappeared.

Specific activity of intestinal enzymes in post larvae from CF treatment was slight higher at 60 DAH than the activity in post larvae from SW treatment, suggesting that a co-feeding regime has a lower effect on intestine maturation. Both alkaline phosphatase and aminopeptidase showed an increase on activity at 60 DAH, showing that at this age post larvae had an increase in intestine renewal. The increase in alkaline phosphatase and aminopeptidase activities corresponds to maturation of enterocytes and digestion in the brush border (Ribeiro *et al.,* 2002; Suzer *et al.*, 2007).

Total activity showed that there were no differences in enzymatic activity independently of the treatment, proving that in this experiment none of the treatments influenced differently the enzymatic activity.

Enterocyte maturation index decreased in both enzymes and treatments at 60 DAH, the exception was aminopeptidase for post larvae of SW treatment. The decrease in enterocyte maturation could be a direct response to what was visible in post larvae physical condition. By the end of the experimental period there were no significant differences between treatments concerning the maturation index, in opposition to results obtained at 40 DAH. This might indicate that post larvae at this age were no further affected by the type of weaning used or reflects the low physical condition of post larvae from both treatments, because of the decrease in the enterocyte maturation. Nevertheless the decrease on the enterocyte maturation, the present work obtained better results than the ones reported by Ribeiro *et al.* (2002). This author obtained at 46 DAH with sole fed artemia or ICES diet enterocyte maturation of 14.3 % and 7.1 % respectively, calculated using alkaline phosphatase.

In conclusion:

- Besides the results obtained with growth, survival and total activity that reflected post larvae low physical conditions, the specific activities were similar with the ones obtained in other studies with sole with the same age.

- The weaning method that seemed to less affect the development and enzymatic activity was sudden weaning, being this the most desirable method to use in production, since it introduces the feed earlier avoiding further utilization of live feed.

- The enzymatic activity seemed not be affected by the type of weaning, and if it was affected the level of activity by the end of the experimental period was similar for both types of weaning.

- Except for the enzymes analysed on the brush border that could suggest co-feeding regime as a better weaning strategy, all other enzymes activities indicated that sudden weaning was better strategy.

- The study of digestive enzymatic activity, specially the total activity, allowed seeing the physical condition of post larvae and the effects of the weaning strategy.

- The present work confirmed the possibility of weaning larvae with 30 DAH and with a lower weight than the one reported in other studies, nevertheless with poor results.

- The results obtained with this experiment could be affected by the post larvae low weight used since the beginning of the experiment.

- With the results obtained in this study it is not possible to conclude what is the better method of weaning because both weaning strategies produced poor results.

Further multidisciplinary studies are needed to really understand the better method of weaning and the effects of weaning in sole post larvae.

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