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Efeito da dieta no desenvolvimento larval do peixe-rei *Odontesthes argentinensis* (Valenciennes, 1835): aspectos histológicos, bioquímicos e fisiológicos

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O conhecimento de nossa história ao longo da evolução revela que todos os organismos vivos, desde as bactérias até o ser humano descendem de um antepassado comum. Este conhecimento possibilita a contextualização de nossas ações no presente.

Ao longo da evolução humana, nossos ancestrais desceram das árvores e ocuparam um novo ambiente, as savanas, com novos recursos alimentares; o que permitiu o aumento do grupo neste novo ambiente. Com uma maior competição intra-especifica tivemos que desenvolver a capacidade de nos comunicar, para conseguir explicar o mundo a nossa volta, a fim de reforçar o relacionamento entre os membros do bando. Para isso desenvolvemos cérebros maiores capazes de processar de forma mais complexa esta nova visão do mundo. O polegar opositor juntamente com a postura bípede nos permitiu ficar com as mãos livres, passando posteriormente a utilizar ferramentas. Este conjunto de fatores, do acaso da evolução fez com que nos tornássemos seres racionais, '*Prometeu entregara a chama para nós'*.

Somos hoje homens pensantes capazes de transformar a natureza a nossa volta. Ao longo do tempo, cultivamos o solo, domesticamos animais, porém as necessidades vitais continuaram as mesmas: alimentar-se e dar continuidade à espécie, tornando esta busca continua.

Atualmente gozamos de grande sucesso na criação de animais, embora apenas um pequeno número tenha sido domesticado com sucesso. A domesticação de novas espécies depende das características desta, tornando necessário o desenvolvimento de novas tecnologias. Hoje, o ambiente marinho que para alguns poderia ser o mais improvável de ser cultivado, é talvez a ultima fronteira na terra, nesta busca incessante por novas fontes de alimento.

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I. RESUMO

Este estudo avaliou os efeitos do alimento vivo ou inerte na sobrevivência, crescimento, biomassa, organogênese (trato digestório), atividade da tripsina e conteúdo protéico corporal em larvas do peixe-rei *Odontesthes argentinensis* entre 2 e 32 dias após a eclosão (dae). As larvas foram submetidas à 5 regimes alimentares: náuplios de *Artemia* (NA) por 30 dias; NA por 15 dias e alimento artificial (AA) por 15 dias; NA por 10 dias e AA por 20 dias; NA por 5 dias e AA por 25 dias. Após 32 dae, a sobrevivência, o crescimento e a biomassa foram dependentes do regime alimentar, diminuindo com o aumento da duração do tratamento das larvas com alimento artificial. Ao longo do desenvolvimento ontogenético, larvas que se alimentaram exclusivamente de NA mostraram uma tendência em acumular inclusões protéicas nos enterócitos, enquanto uma maior ocorrência de inclusões lipídicas foram observadas naquelas alimentadas com AA. A atividade corporal da tripsina foi menor nas larvas alimentadas exclusivamente com NA do que naquelas alimentadas exclusivamente com AA. Uma resposta contrária foi observada para a concentração corporal de proteínas. Os dados obtidos indicam que as larvas de peixe-rei são capazes de ingerir dieta inerte como primeiro alimento e que taxas de sobrevivência e crescimento relativamente altas podem ser obtidas com uma dieta mista de NA e AA. Entretanto, uma biomassa 9 vezes maior foi obtida quando as larvas foram alimentadas exclusivamente com NA do que com AA. Tal diferença parece resultar de uma melhor eficiência do trato digestório em digerir e acumular proteínas nas larvas alimentadas exclusivamente com NA, refletindo em uma maior acumulação corporal de proteínas e um maior crescimento nestas larvas.

Palavras-chave: crescimento, dieta, *Odonthestes argentinensis*, organogênese, peixe-rei, proteína, sobrevivência, tripsina.

II. INTRODUÇÃO

O cultivo intensivo de larvas de peixes aparece como uma alternativa interessante para se maximizar a produção de juvenis de qualidade para a piscicultura, através da redução da mortalidade durante esta fase crítica da produção. Neste sistema, as larvas são cultivadas em laboratório recebendo alimentos mais adequados sem a presença de predadores (Jomori, 2001). Atualmente, a larvicultura intensiva de peixes é aplicada por alguns produtores no Brasil para o cultivo de espécies carnívoras que possuem alto valor econômico (Jomori et al., 2003).

A produção intensiva de larvas de peixes e crustáceos está baseada, quase que exclusivamente, na utilização de alimentos vivos, tais como rotíferos e náuplios de *Artemia*. No entanto, alguns inconvenientes têm sido reportados em relação ao cultivo de alimento vivo em larga escala. Dentre estes, cita-se os altos custos para a aquisição dos cistos de *Artemia* e aqueles relacionados com a mão de obra para a sua produção; a possibilidade de introdução de bactérias que podem contaminar o cultivo e serem nocivas às larvas; o valor nutricional das presas em função do local geográfico da coleta, e ainda, o risco de um colapso no cultivo que pode interromper o suprimento de alimento vivo a qualquer momento (Lee et al., 1997).

A utilização de dietas inertes, o mais precocemente possível, na alimentação de larvas de peixes e crustáceos é, portanto, de extremo interesse e uma promissora solução para o futuro (Hart e Purser, 1996; Önal e Langdon, 2000). Por exemplo, Ehrlich et al. (1989) citam os benefícios da utilização de dieta inerte na alimentação de larvas de *Micropterus dolomieui*, reduzindo os custos com mão de obra em cerca de 60%, comparativamente com a produção das larvas exclusivamente feita com *Artemia*. No entanto, segundo Sorgeloos et al. (2001), nenhuma dieta inerte pode substituir

completamente o alimento natural. Para solucionar tal problema, a utilização de enzimas exógenas tem sido sugerida (Walford e Lam, 1993; Kolkovski et al., 1993). Recentemente, Tesser (2005) suplementou uma dieta inerte com a pancreatina de porco, resultando em maior crescimento final e sobrevivência de larvas de pacu *Piaractus mesopotamicus*, corroborando os resultados obtidos por Kolkovski et al. (1993).

No entanto, segundo Cahu e Zambonino Infante (2001), apesar das larvas de peixes possuírem enzimas digestórias, o aparecimento da função digestória está associada a transformações morfológicas, que seguem uma seqüência cronológica nas larvas em desenvolvimento, assim como ocorre nos mamíferos. Segundo Verreth e Segner (1995), os peixes passam por adaptações evolutivas na morfogênese de seu sistema digestório durante o desenvolvimento inicial, devido principalmente às mudanças nas exigências nutricionais e do tipo de alimentação, à qual deixa de ser endógena e passa a ser exógena. Portanto, estes fatores se refletem na ontogenia dos padrões de surgimento enzimático. Boulhic e Gabaudan (1992) citam que a principal alteração é a diferenciação do estômago. De fato, alguns autores relatam que o momento de substituição do alimento vivo pelo inerte ocorre quando o estômago é funcional (Segner et al., 1993; Kolkovski, 2001). Porém, a sua utilização continua sendo fundamental nas larviculturas comerciais, sendo que os insucessos da utilização de dietas inertes para a primeira alimentação de larvas de peixes têm sido atribuídos, principalmente, a um deficiente sistema de enzimas digestórias nos estágios larvais.

Com base no exposto acima, denota-se que estudos devem ser realizados na tentativa de associar as necessidades nutricionais dos peixes com o tipo de alimentação a ser fornecida e o sistema digestório da espécie em cultivo. Segundo Kolkovski e Dabrowski (1999), as necessidades nutricionais das larvas, tanto do ponto de vista qualitativo como quantitativo, são distintas daquelas dos adultos e varia ao longo do

desenvolvimento larval. Portanto, a análise da ontogenia do sistema digestório, incluindo estudos morfológicos e enzimáticos em peixes, é extremamente importante para o estabelecimento de protocolos alimentares e até mesmo nutricionais, reduzindo assim sensivelmente o manejo para a produção de larvas e juvenis, bem como os custos finais de produção (Tramati et al., 2005).

No que se refere aos aspectos enzimáticos nas larvas de peixes, Appelbaum e Holt (2003) determinaram a importância do estudo da atividade da quimiotripsina, uma vez que foi observada uma relação entre esta enzima e a condição nutricional de larvas de *Sciaenops ocellatus*. Além disso, Lemieux et al. (1999) demonstraram que a atividade da tripsina pode potencialmente limitar o crescimento de juvenis do bacalhau *Gadus morhua*. Normalmente, as atividades das diferentes enzimas do trato digestório seguem uma seqüência cronológica de aparecimento. Por exemplo, Walford e Lam, (1993) observaram ma redução da atividade da tripsina ao longo do desenvolvimento larval, tendendo a zero após 30 dias de vida em *Lates calcifer*.

No que se refere aos aspectos morfológicos, o estudo do desenvolvimento ontogenético larval de teleósteos possibilita o estabelecimento da evolução gradual dos órgãos e sistemas. No caso do sistema digestório, as transformações são constantes e ocorrem rapidamente nos primeiros dias após a eclosão das larvas. O trato digestório das larvas é morfo-fisiologicamente menos elaborado do que o dos adultos. O seu desenvolvimento ocorre concomitantemente com o crescimento larval e passa por complexas mudanças, ou seja, a larva passa por um modelo de ontogenia trófica, ocorrendo trocas na dieta com o processo de crescimento. Como resultado destas trocas, observa-se uma diferenciação das necessidades nutricionais (Lavens *et al*., 1996).

Logothetis et al. (2001) estudando larvas de *Atherinops affinis*, um Atherinopsidae do Atlântico Norte, demonstrou que estas eram desprovidas de um

estômago funcional e possuíam hábito alimentar onívoro, com tendência a herbivoria. O peixe-rei *Odonthestes argentinensis*, um Atherinopsidae do Atlântico Sul, também apresenta hábito onívoro, alimentando-se de plâncton e detritos (Fisher et al., 2004). Costa (2001) estudando o desenvolvimento morfológico larval desta espécie verificou que suas larvas eclodem com a maioria dos órgãos indiferenciados. O pâncreas e o fígado aparecem como pequenos agrupamentos celulares ao lado do vitelo. Ao eclodir, essas larvas já apresentam olhos pigmentados, sistema digestório aberto (boca e ânus) e vesícula gasosa inflada. No entanto, até o 30º dia após a eclosão, um estômago morfológico não foi observado.

O peixe-rei *O. argentinensis* se distribui na região costeira do Atlântico Sul, penetrando nos estuários, ambientes aos quais está perfeitamente adaptado, sendo considerada uma espécie estuarina residente (Chao et al., 1985). O interesse pela piscicultura na zona estuarina e costeira do Sul do Rio Grande do Sul, bem como as questões ambientais relacionadas à introdução de espécies exóticas, têm exigido a busca da identificação de espécies locais que apresentem potencial de aproveitamento para cultivo, sendo que as espécies de peixe-rei, como *O. bonariensis*, despertam interesse (Piedras, 2003). Diversos trabalhos também têm demonstrado que o peixe-rei *O. argentinensis* apresenta características biológicas que o tornam uma espécie potencialmente cultivável (Phonlor e Vinagre, 1989; Ostrensky e Brugger, 1992; Phonlor e Sampaio, 1992; Sampaio, 1992; Sampaio et al., 1995; Sampaio e Minillo, 1995; Sampaio e Phonlor, 1996). Portanto, o amplo conhecimento da biologia destas espécies é fundamental para o aproveitamento destes recursos piscícolas em aqüicultura. No entanto, a literatura relacionada a organogênese e ao desenvolvimento larval do peixe-rei *O. argentinensis* é escassa (Phonlor e Cousin, 1998; Costa, 2001). Sabe-se que as larvas desta espécie podem iniciar sua primeira alimentação diretamente com dieta

inerte. Porém, o crescimento observado é reduzido quando comparado àquele das larvas alimentadas com náuplios de *Artemia* (Sampaio et al. 1995). Até o presente momento, nenhum estudo foi realizado na tentativa de relacionar o tipo de alimentação oferecida (viva ou inerte) com a organogênese e a atividade da tripsina em *O. argentinensis*.

III. OBJETIVOS

Objetivo geral

O objetivo central deste estudo foi contribuir para o conhecimento do desenvolvimento do trato digestório do peixe-rei marinho *Odontesthes argentinensis* e a possível influência da dieta nesse processo.

Objetivos específicos

- Determinar a sobrevivência de larvas de peixe-rei submetidas a diferentes protocolos alimentares utilizando-se dieta viva (náuplius de *Artemia spp.*) ou inerte (ração).
- Analisar o crescimento de larvas de peixe-rei submetidas aos diferentes protocolos alimentares utilizados.
- Avaliar os efeitos da dieta na organogênese do trato digestório de larvas de peixe-rei.
- Determinar os efeitos da dieta na atividade tríptica e concentração de proteínas em nível corporal nas larvas de peixe-rei.

IV. ARTIGO

Influence of live and inert diet on larval development of the marine silverside *Odontesthes argentinensis* (Valenciennes, 1835)

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Artigo a ser submetido à revista AQUACULTURE

Influence of live and inert diet on larval development of the marine silverside *Odontesthes argentinensis* (Valenciennes, 1835)

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Abstract

This study evaluated the effect of inert and live food on survival, growth, biomass, organogenesis (digestive tract), whole body trypsin activity and protein content in larvae of the silverside *Odontesthes argentinensis* from 2 to 32 days post hatching (dph). Silverside larvae were subjected to five different feeding regimes: *Artemia* nauplii (AN) for 30 days; AN for 15 days and artificial dry food (AF) for 15 days; AN for 10 days and AF for 20 days; AN for 5 days and AF for 25 days; and AF for 30 days. Survival, growth and biomass at 32 dph were dependent on the feeding regime, decreasing with the increased duration of larvae treatment with AF. Over the ontogenetic development, larvae fed exclusively on AN showed a tendency to accumulate protein inclusions in enterocytes, while a higher occurrence of lipid inclusions was observed in those fed on AF. Whole body trypsin activity was significantly lower in larvae fed exclusively on AN than in those fed exclusively on AF. An opposed situation was observed for the whole body protein concentration. Taken together, these findings indicate that silverside larvae is capable to eat inert diet as first feeding and that relatively high survival and growth rates can be obtained with a mixed diet of AN and AF. However, a 9-fold higher biomass was obtained when larvae were fed exclusively on AN than AF. Such difference seems to be a result of a better efficiency of the digestive tract to digest and accumulate proteins, reflecting in a markedly higher whole body accumulation of proteins and growth in AN-fed larvae.

Keywords: feeds, growth, larvae, organogenesis, *Odontesthes argentinensis*, protein, silverside, survival, trypsin activity

1. Introduction

Live food is still considered as essential for the first feeding of marine fish larvae because it increases fish feeding, stimulates enzyme secretion, and results in adequate fish growth and survival (Hart and Purser, 1996; Önal and Langdom, 2000). However, cost associated with its use in aquaculture is generally high. For example, rearing of large yellow croaker larvae and juveniles still depends totally on live prey, which accounts for more than 70% of the total cost for 40-days old juveniles (Hongming et al., 2005). Other problems associated with the use of live food in aquaculture are the possibility of fish and/or water contamination with bacteria, variability of the nutritional value of preys according to the collection site, and risk of culture collapse by interruption of live food availability at any moment (Lee et al., 1997).

Many of the problems pointed above could be solved with the development of a dry diet formulated to fulfill the nutritional requirements of the larvae. However, fish behavior and physiological capabilities will determine whether larvae will eat or not the manufactured diet (Chang et al., 2006). At this point, a critical stage in fish culture is the weaning of larvae from live to formulated feeds. The limited success of marine larviculture is generally due to an inadequate knowledge of the functional development of the digestive system, as well as the nutritional requirements of the larvae (Hamlin et al., 2000; Mai et al., 2005).

The digestion mechanisms in fish larvae have been particularly studied during the last two decades as a means of understanding the nutritional needs of fish and the effect of dietary constitutes on enzyme activity (Zambonino Infante and Cahu, 2001). In turn, histological parameters have been suggested as useful indicators of fish nutritional

status (Storch and Juario, 1983; Strüssmann and Takashima, 1990). Analysis of digestive enzymes activities has been also indicated as an easy and reliable methodology that can be used to evaluate the digestive processes and nutritional condition of fish larvae (Ueberschär, 1988).

The ontogenetic development capability of the digestive tract to secrete digestive enzymes in marine fish larvae feeding on dry food has been studied by many authors (Nolting et al., 1999; Lazo et al., 2000; Chen et al., 2006). The tryptic activity has been shown to be important to digestion, particularly because of its role in activating the other alkaline proteases (Appelbaum and Holt, 2003). For example, Lemieux et al. (1999) reported that among the several enzymes tested, trypsin was the only one related to food conversion efficiency in the adult Atlantic cod *Gadus morhua*. They also suggested that trypsin plays an important role in the growth regulation of this species.

The marine silverside *Odontesthes argentinensis* is an omnivorous Atherinopsidae fish feeding on plankton as juvenile and on benthos as adult (Bemvenuti, 1990). In Brazil, it is found along the Southern Atlantic coast, including estuaries, being considered an estuarine resident species (Chao et al., 1985). Several studies have pointed the potential of this species for aquaculture in Southern Brazil (Phonlor and Vinagre, 1989; Ostrensky and Brugger, 1992; Sampaio, 2006). However, no studies on the effects of different feeds on the digestive tract organogenesis and physiology of *O. argentinensis* are available.

In light of the above, the objective of the present study was to evaluate the effects of different feeding protocols on survival, growth and histological features of the digestive tract, as well as on whole body tryptic activity and protein concentration in larvae of *O. argentinensis*. Larvae were cultivated for 32 days post hatching and were fed on five different combinations of live and inert feeds.

2. Materials and methods

2.1. Egg collection and hatching

Larvae of the marine silverside *Odontesthes argentinensis* (Valenciennes, 1835) were obtained from eggs caught at the Cassino Beach ($32^0$12'S; $52^0$10'W, Rio Grande, RS, Southern Brazil). Eggs collected were transferred to our Aquaculture Marine Station, stored and incubated in 40-L plastic tanks containing seawater (28 ppt) at 20°C, which was continuously aerated. Egg maintenance and hatching were performed according to Phonlor and Vinagre (1989).

2.2. Experimental conditions

After 2 days post hatching (dph), 430 larvae were stocked in a plastic tank containing 50 L of seawater. Three tanks were tested per treatment, as described below. They were arranged as an opened circulating system with water exchange at the rate of 8 L h^{-1} and cleaned daily by siphoning the bottom to remove dead larvae and deposed organic matter. Mean water salinity and temperature over the whole experiment (32 days) was 27.6 ± 0.6 ppt and 23.2 ± 1.4°C, respectively. Photoperiod was fixed at 18L:6D.

Larvae were fed four times a day for 30 days. They were subjected to one of the following five feeding protocols: (A) larvae fed only *Artemia spp.* nauplii (INVE Americas, Salt Lake City, UT, EUA); (B) larvae fed 5 days on *Artemia spp.* nauplii and 25 days on dry food; (C) larvae fed 10 days on *Artemia spp.* nauplii and 20 days on dry

food; (D) larvae fed 15 days on *Artemia spp.* nauplii and 15 days on dry food; (E) larvae fed only dry food (Fig. 1). The protocol A corresponded to the control treatment. Each protocol was tested in triplicate.

In the beginning of the experiment (2 dph), *Artemia spp.* nauplii were offered at a final density of 1 nauplius mL^{-1}. However, it increased over the experiment reaching 32 nauplii mL^{-1} at the end of experiment (32 dph). Artificial food was offered at 1g $tank^{-1}$ at the beginning of the experiment and was increased up to 3.5 g $tank^{-1}$ at the end of the experiment. Artificial food included three commercial microdiets (INVE Americas, Salt Lake City, UT, EUA): Proton 2 (150-300 µm), NDR 4/6 (400-600 µm) and NDR 5/8 (500-800 µm) (Fig.1).

2.3. Survival and growth

After 2 dph, thirty larvae (0.81 ± 0.04 cm; 2.5 ± 0.3 mg) were sampled from the incubation tank before larvae stocking at the experimental tanks. Every five days of experiment (7, 12, 17, 22, and 27 dph), one larvae sample (n = 10) was randomly collected from each experimental tank (3 samples per treatment; n = 30). At the end of the experiment (32 dph), surviving larvae in each tank were counted and 20 larvae per tank were randomly sampled. Collected larvae were anesthetized with tricaine methanesulphonate (MS 222; 50 ppm), blotted dried, weighed (wet weight; g), measured (total length; cm), fixed in Bouin solution for 12-24 h, and stored in 70% alcohol for histological analysis, as described below.

Larvae survival in each tank was calculated based on the total number of expected surviving larvae at the end of the experiment (32 dph), *i.e.*, 380 larvae. It must be considered that 430 larvae were initially stocked in each tank (2 dph) and that 10

larvae were sampled per tank at 7, 12, 17, 22 and 27 dph, totalizing 50 sampled larvae per tank over the experimental time.

Growth was reported as specific growth rate (SGR; change in body weight per day) and was calculated from the average body weight as follows: SGR (%) = $ln\ (Wf) - ln(Wi)/t\ x\ 100$, where W_i and W_f are respectively the initial and final wet weights (g) within the considered time period, and *t* is the time period (days). Biomass (g) in each tank was calculated as follows: $B = mWf\ x\ N$, where *mWf* is the mean final wet weight of larvae and *N* is the number of surviving larvae after 32 dph in the respective tank.

2.4. Histological analysis procedure

Histological features of larval digestive tract were analyzed in larvae collected and stored as described above. Two larvae collect from each treatment at the beginning (2 dph) and after 5 (7 dph), 10 (12 dph), 15 (17 dph), 20 (22 dph), and 25 days of experiment (27 dph) were analyzed. At the end of the experiment (32 dph), four larvae collected from each treatment were analyzed.

All samples stored in 70% alcohol were dehydrated through a standard series of increasing alcohol concentrations, cleared with xylene, and embedded in paraffin. Blocks were sliced following a series of 7-µm sagittal sections. Sections were mounted onto glass slides, stained with haematoxylin and eosin, and observed under a light microscopy (JENAMED 2, Carl Zeiss, Germany).

After 2 dph, a general morphological and histological description of the larvae digestive tract was performed. Over the experimental time, histological features were evaluated considering the morphology and structure of liver, intestine and pancreas. Enterocyte length and hepatocyte nucleous diameter were also measured in larvae from all treatments. Enterocyte length was measured using an ocular microscale from the

optic microscope (JENAMED 2, Carl Zeiss, Germany) while the hepatocyte nucleous diameter was measured using imaging analysis (UTHSCSA ImageTool, Version 3.0, University of Texas, San Antonio, TX, USA). Thirty cells per larvae were analyzed.

2.5. Trypsin assay

On day 32 dph, feeding was stopped for 12 h and then three larvae from each tank (9 larvae/treatment) were randomly sampled, killed with an overdose of MS 222, transferred to plastic tubes, immediately frozen in liquid nitrogen, and stored at –80°C until enzymatic activity analysis, as described below.

Whole larvae were individually homogenized (1 mg sample/2 ml buffer) in a Potter-microhomogenizer using cold Tris-HCl buffer (0.1 M, pH 8.0) containing $CaCl_2$ (0.02 M). Homogenate was centrifuged at 6,000 *x* g for 60 min at 4°C (Micro22R, Hettich Zentrifugen, Global Medical Instrumentation, Ramsey, MN, USA). The supernatant was used as enzyme source and for protein content analysis. Tryptic activity measurement was performed using a fluorescence technique adapted from Ueberschär (1988). Reaction was performed with 250 µL of Nα-carbobenzoxy-L-arginine-4-methylcoumarinyl-7-amide (CBZ-L-Arg-MCA) solution (0.2 mM) as substrate and 10 µL of homogenate. Increase in fluorescence emission at 440 nm (excitation at 380 nm) was measured every 2 min intervals up to 60 min (Victor 2, Perkin-Elmer, Waltham, MA, USA). Only the linear portion of the fluorescence response over time was considered for enzyme activity calculations. Trypsin activity was normalized by the protein concentration in the supernatant. Protein was determined using a commercial reagent kit (Proteínas Totais®; Doles, Goiânia, GO, Brazil) based on the Biuret assay.

Trypsin activity was expressed as μg trypsin/g proteins. Whole body protein content was expressed as mg proteins/g body wet weight.

2.6. Data and statistical analyses

Data were expressed as mean ± SD. Differences between treatments were evaluated by one-way analysis of variances (ANOVA) followed by the Tukey test. The significance level adopted was 95% (α=0.05). ANOVA assumptions were previously checked. Survival and tryptic activity data were previously transformed (arcsin and log transformation, respectively) to meet ANOVA assumptions.

3. Results

3.1. Growth and survival

Survival, total length, wet weight, SGR, and biomass data of 32-dph larvae are shown in Table 1. Survival was considerably high in all treatments (78-94%), except in larvae fed only artificial food (54%). The highest survival rate (94%) was observed in larvae fed only *Artemia* nauplii. The total length, wet weight, SGR and biomass of larvae fed only *Artemia* nauplii were significantly higher than those of larvae fed only artificial food or combined diets. The lower values were always observed for larvae fed only artificial food (Table 1). Significant differences in growth (total length or wet weight) between larvae fed only *Artemia* nauplii and only artificial food were clearly observed after 12 dph (Tables 2 and 3).

3.2. Histological and cytological examination

Changes in intestinal and hepatic features were observed during the larval development. These differences are described below according to the larvae age.

2 dph- The digestive system was already differentiated in buccopharynx, esophagus, liver, pancreas and intestines. The yolk sac was not totally absorbed (Fig. 2A). The mouth was opened. The hepatic structure was well developed and contained polyhedral hepatocytes in cordon-like arrangement. Hepatocytes had homogeneous eosinophilic cytoplasm, characteristic nucleus and nucleolus, and very well-developed endoplasmatic reticuli. Pancreatic acini showed zymogen granules within the cytoplasm. Intestinal mucosa showed villosity and microvilli as a distinct brush-border.

7 dph- Larvae fed only *Artemia* nauplii showed hepatocytes with low occurrence of lipid inclusions. Their pancreas showed low occurrence of zymogene granules (Fig. 2B). The posterior intestine contained protein inclusions resembling eosinophilic crystal-like structures within the apical enterocyte region. Larvae fed exclusively on artificial food also showed hepatocytes with lipid deposits (Fig. 2C). Their pancreas contained zymogene granules and the posterior intestine had lipid vacuoles.

12 dph – Larvae fed exclusively on *Artemia* nauplii showed more occurrence of pancreatic lipid deposits than those fed exclusively on artificial food. The intermediate and posterior intestine contained more protein than lipid vacuoles. However, larvae fed exclusively on *Artemia* nauplii showed similar hepatic structure compared to those subjected to the other treatments (Fig 2D). Larvae fed exclusively on artificial food showed augmented lipid deposits in the liver and higher occurrence of lipid vacuoles in the posterior intestine (Fig 2E). Their pancreas showed absence of lipid deposits and less zymogene granules than those subjected to the other diets.

17 dph – Hepatic and pancreatic structure did not change between treatments. The structural arrangement observed for each organ remained unchanged up to the end of the experimental period. Larvae from all treatments had a pancreas containing inter-acinar lipid tissue (Fig. 2F). The posterior intestine of larvae fed exclusively on artificial food showed a lower occurrence of lipid vacuoles and higher occurrence of protein vacuoles compared to what was observed in larvae from the same treatment at 12 dph. On the other hand, larvae fed exclusively on *Artemia* nauplii contained only protein vacuoles, while those fed 20 or 25 days on artificial food contained only lipid vacuoles (Fig. 2G).

22 dph – The posterior intestine of larvae fed exclusively on artificial food or fed 20 days with this feed showed lipid vacuoles, while those fed exclusively on *Artemia* nauplii contained protein vacuoles. Larvae fed 15 days on artificial food contained both kinds of vacuoles.

27 dph – Posterior intestine of larvae fed exclusively on artificial food or fed 15 or 20 days on this feed contained lipid vacuoles (Fig. 2H), while those fed only *Artemia* nauplii did not show any vacuoles (Fig. 2I).

32 dph – Larvae from all treatments showed a similar histological structure of the digestive tract. Lipid and protein vacuoles were absent in the posterior intestine (Fig. 2J).

3.3. Hepatocyte morphology

In a broad view, the mean nuclear size of hepatocytes was similar in all treatments at each experimental time. The only exception were larvae fed 15 days and

20 days artificial food, which had hepatocytes with bigger nuclear sizes at 17 dph and 22 dph, respectively (Table 4).

3.4. Enterocyte length

Significant differences in enterocyte length between treatments were observed only at 32 dph. At this experimental time, larvae fed only artificial food showed significantly shorter enterocytes than those fed only *Artemia* nauplii or those fed 15 days artificial food (Table 5).

3.5. Whole body trypsin activity and protein concentration

Whole body tryptic activity was analyzed only at the end of the experiment (32 dph). Larvae fed artificial food showed significantly ($P<0.05$) higher enzyme activity than those fed only *Artemia spp.*, while those from the other treatments had intermediate values (Fig. 3).

Whole body protein content of larvae fed only artificial food was significantly ($P<0.05$) lower than that of those subjected to the other treatments (Fig. 4).

4. Discussion

In the present study, larvae of the silverside *Odontesthes argentinensis* were cultivated for 30 days (2 to 32 dph) under five different feeding regimes combining live (*Artemia* nauplii) and inert (dry artificial food) feeds. Data obtained from survival and growth studies are in complete accordance with those previously reported for the same

species (Sampaio, 2006). They clearly indicate that silverside larvae were able to eat dry artificial food as first feeding. This statement is based on the following facts: (1) a relatively important survival rate (54%) was observed in larvae feeding exclusively on dry artificial food from 2 to 32 dph; (2) a complete development of the digestive tract was observed in all feeding treatments; (3) a significant growth (wet weight and length) over the experimental time was observed in all treatments.

Survival rate as high as 90% was previously reported in delayed feeding trials (8.5 dph) with *O. argentinensis* larvae (Phonlor and Vinagre, 1989). Thus, the reduced survival observed in larvae fed exclusively on artificial food dried cannot be ascribed to the time of first feeding adopted in the present study (2 dph), but to the quality of the food offered to larvae. Furthermore, Strüsman and Takashima (1989) showed that abnormal hepatic arrangement is a good indicator of starvation in silverside larvae. In this case, shrinkage of the hepatocyte nucleus is observed in starved larvae (Strüsman and Takashima, 1990). In fact, this cell type is particularly useful as an indicator of fish nutritional status (Storch et al., 1983; Storch and Juario, 1983). It is also known that a slight disorganization of the pancreatic acinar arrangement can be induced by starvation periods as short as one day (Strüsmann and Takashima, 1989). In the present study, no abnormal hepatic or pancreatic histological arrangements were observed at any feeding treatment over the experimental time. Taken all together, these findings clearly suggest that *O. argentinensis* larvae did not starve over the experimental time in the present study.

Although silverside were able to feed on artificial food as first feeding, data obtained in the present study showed that survival and growth rates were quite dependent on the feeding regime adopted. Larvae feeding on *Artemia* nauplii for at least 5 days (2 to 7 dph) showed high survival rates (>78%), indicating that *Artemia* nauplii

wood be much more adequate as first feeding for proper larvae development than artificial dry food. Similar results were observed for larvae of the red drum *Sciaenops ocellatus* (Lazo et al., 2000).

Despite the high survival rates observed in larvae feeding on *Artemia* nauplii for at least 5 days, significant lower growth rates (weight and length) were observed when they were fed dry artificial food. Decrease in growth rate was inversely related to the duration of the feeding treatment with this feed. As a consequence, biomass observed after 30 days of experiment (32 dph) was approximately 2, 3, 4 or 9-fold higher in larvae feeding exclusively on *Artemia* nauplii than in those feeding on dry artificial food for 15 (17 to 32 dph), 20 (12 to 32 dph), 25 (7 to 32 dph) or 30 days (2 to 32 dph), respectively. This finding suggests a differential ability of the digestive tract to accumulate nutrients, especially proteins, over the experimental time (2 to 32 dph) in larvae subjected to the different feeding regimes. This statement is based on the fact that a clear reduction in whole body protein concentration was observed with the introduction of artificial dry food. Furthermore, the degree of reduction was directly proportional to the duration of the treatment with artificial food, being approximately 50% in larvae fed exclusively on artificial dry food when compared to those fed exclusively on *Artemia* nauplii. This finding suggests a differential development of the ability to digest and accumulate protein from the food in larvae subjected to the different feeding treatments.

The use of whole body tryptic activity as an indicator of larval condition and rate of development was evaluated by Ueberschär (1988, 1995). In fact, trypsin secretion in marine fish can be modulated by dietary protein content and level of food intake (Pedersen et al., 1990; Cahu and Zambonino Infante, 1994; Zambonino Infante and Cahu, 2001). A decreased tryptic activity was observed in larvae of the yellowtail

kingfish *Seriola lalandi* (Chen et al, 2006) and other marine fish species (Zambonino Infante and Cahu, 2001) when fed on live food. In the present study, a significantly higher tryptic activity was also observed in silverside larvae fed exclusively on artificial dry food than in those fed exclusively on *Artemia* nauplii. This finding suggests that the higher trypsin secretion, and a the consequently higher enzymatic activity observed in larvae feeding on artificial dry food, would be a larval strategy to compensate a lower quality of the food supplied or a possible delay in the development of the digestive tract capability to digest and accumulate proteins. In fact, it has been demonstrated in larvae of the yellowtail kingfish that trypsin activity is higher in the fish larval stage and progressively decreases as the stomach become functional. In this case, a higher SGR of larvae fed exclusively on *Artemia* nauplii was paralleled with a low tryptic activity, demonstrating a complete development of the alimentary tract (Chen et al, 2006). Therefore, the significantly lower tryptic activity observed in larvae of the silverside *O. argentinensis* fed exclusively on *Artemia* nauplii could indicate a proper development of the alimentary tract ability to digest and accumulate proteins. This assumption is supported by the increased whole body levels of tissue proteins observed in these larvae and the histological findings from the present study, as discussed below.

Regarding the development of the digestive tract in the silverside *O. argentinensis*, ontogenetic changes in the digestive system were observed over the experimental time. For example, a normal hepatic structure with lipid deposits was observed in larvae from all treatments. However, these lipid deposits disappeared after 12 dph and a inter-acinar lipid tissue was observed in the pancreas. In goldfish, a normal hepatic structure and similar hepatocytes were also observed at the end of yolk sac resorption when larvae were fed on *Artemia* or mixed diet. However, those fed exclusively on artificial food showed hepatic necrosis (Abi-Ayad and Kestemont, 1994).

The intestine is the longest portion of fish digestive tract (Bisbal and Bengtson, 1994). In the present study, inclusion vacuoles were observed in the posterior intestinal epithelium of larvae of the silverside *O. argentinensis*. These vacuoles were similar to those found in fish larvae that compensate lack of stomach and pepsin digestion with active intracellular digestion in the epithelial cells of the posterior intestine, preceded by picnocytosis of macromolecules from the intestinal lumen (Ostaszewska et al., 2005). In the present study, intestinal cellular structures were similar in larvae from the different feeding treatments. However, a visually higher occurrence of lipid inclusions was observed in enterocytes of larvae fed on artificial dry food while protein inclusions were more frequent in enterocytes of those fed exclusively on *Artemia* nauplii. Furthermore, the latter showed shorter enterocytes than the former. These findings suggest that a proper development of the digestive tract to digest and accumulate proteins is better evidenced in silverside larvae fed exclusively on *Artemia* nauplii, corroborating with the results discussed above on whole body tryptic activity and protein content.

In summary, data from survival studies indicate that 2 dph larvae of the silverside *O. argentinensis* can be fed with dry artificial food as first feeding. Furthermore, they also showed that a compound diet (*Artemia* nauplii and dry artificial food) can be used in the feeding sequence of silverside larvae, resulting in very high survival rates. However, data from digestive tract histology and whole body enzyme activity studies clearly demonstrated that a possible delayed development of the alimentary tract reduced the ability to digest and accumulate protein in larvae fed on artificial dry food. As a consequence, a considerable reduction of larvae growth rate, and consequently of the biomass produced after 30 days of cultivation (32 dph), was observed in those larvae.

Further research is needed to evaluate the nutritional requirements of silverside larvae, since a possible effect of food quality on the results obtained in the present study cannot be ruled out. Future studies should examine the digestibility and assimilation of the different ingredients present in the different feeds employed to better choose the moment to introduce the microparticulate diets in the cultivation of larvae of the silverside *O. argentinensis*.

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Legend to Figures

Figure 1. Feeding protocols used for maintenance and growth of *Odontesthes argentinensis* larvae.

Figure 2. Histological sections of larvae of the marine silverside *Odontesthes argentinensis* fed different feeds. Sections are sagitally oriented and stained with haematoxylin and eosin. A. General view of the digestive tract of 2-dph larvae. B. Liver of 7-dph larvae fed only *Artemia* nauplii. C. Liver of 7-dph larvae fed only artificial food. Note the cellular lipid inclusions (asterisks). D. Posterior intestine of 12-dph larvae fed only *Artemia* nauplii. Note the homogenous eosinophilic feature of the enterocyte cytoplasm. E. Posterior intestine of 12-dph larvae fed only artificial food. Note the lipid cell inclusions (arrows). F. Intestine and exocrine pancreas of 17-dph larvae fed only artificial food. Note the unilocular lipid tissue (asterisks) in the exocrine pancreas tissue and the protein inclusions in the enterocytes (arrows). G. Posterior intestine of 17-dph larvae fed only *Artemia* nauplii. H. Posterior intestine of 27-dph larvae fed 10 days artificial food. Note the large lipid cell inclusions in the enterocytes (arrows). I. Posterior intestine of 27-dph larvae fed only *Artemia* nauplii. Note enterocytes with homogenous eosinophilic apical cytoplasm (arrows). J. Posterior intestine of 32-dph larvae fed only *Artemia* nauplii. Abbreviations: I = intestine; L = Liver; Y = Yolk sac; P = Exocrine pancreas.

Figure 3. Whole body tryptic activity in *Odontesthes argentinensis* larvae. Data are mean ± SD (n = 9). Different letters indicate means significantly different ($P<0.05$) between treatments.

Figure 4. Whole body protein concentration in *Odontesthes argentinensis* larvae. Data are mean ± SD (n = 9). Different letters indicate means significantly different ($P<0.05$) between treatments.

Table 1. Influence of diet on survival (S), total length (TL), wet weight (WW), specific growth rate (SGR), and biomass (B) of *Odontesthes argentinensis* larvae after 32 dph. Data are expressed as mean ± SD. Different letters indicate mean significantly different (P<0.05) between diets for the same parameter analyzed. 15AF, 20AF, 25AF, and 30AF correspond to larvae fed for 15, 20, 25 and 30 days with artificial food, respectively.

	Artemia	15AF	20AF	25AF	30AF
S (%)	93.6 ± 1.4 a	88.7 ± 5.0 ab	88.3 ± 1.5 ab	78.3 ± 5.0 b	53.7 ± 6.9 c
TL (cm)	2.29 ± 0.25 a	1.81 ± 0.43 b	1.60 ± 0.18 c	1.58 ± 0.37 c	1.33 ± 0.17 d
WW (mg)	105 ± 35 a	55 ± 21 b	40 ± 14 bc	34 ± 15 bc	20 ± 8 c
SGR (%)	12.3 ± 1.2 a	10.1 ± 1.6 b	9.1 ± 1.2 bc	8.8 ± 1.5 c	6.7 ± 1.6 d
B (g)	37.2 ± 4.4 a	18.7 ± 5.3 b	13.5 ± 2.1 b	10.2 ± 1.3 bc	4.2 ± 1.1 c

Table 2. Influence of diet on total length (cm) of *Odontesthes argentinensis* larvae up to 32 dph. Data are expressed as mean ± SD (n = 30). Different letters indicate mean significantly different (P<0.05) between diets for the same experimental time. 15AF, 20AF, 25AF, and 30AF correspond to larvae fed for 15, 20, 25 and 30 days with artificial food, respectively.

Time (dph)	*Artemia*	15AF	20AF	25AF	30AF
7	0.97 ± 0.03 a	0.93 ± 0.06 b	0.96 ± 0.04 ab	0.96 ± 0.03 ab	0.93 ± 0.08 b
12	1.12 ± 0.08 a	1.06 ± 0.09 ab	1.12 ± 0.08 a	1.10 ± 0.05 ab	1.05 ± 0.09 b
17	1.38 ± 0.09 a	1.29 ± 0.09 b	1.25 ± 0.09 bc	1.23 ± 0.07 c	1.19 ± 0.9 c
22	1.64 ± 0.12 a	1.42 ± 0.14 b	1.46 ± 0.11 b	1.32 ± 0.12 c	1.39 ± 0.11 c
27	2.06 ± 0.23 a	1.54 ± 0.13 b	1.51 ± 0.10 b	1.40 ± 0.14 c	1.13 ± 0.12 c
32	2.29 ± 0.25 a	1.81 ± 0.42 b	1.60 ± 0.18 c	1.58 ± 0.34 c	1.32 ± 0.16 d

Table 3. Influence of diet on wet weight (mg) of *Odontesthes argentinensis* larvae up to 32 dph. Data are expressed as mean ± SD (n = 30). Different letters indicate mean significantly different (P<0.05) between diets for the same experimental time. 15AF, 20AF, 25AF, and 30AF correspond to larvae fed for 15, 20, 25 and 30 days with artificial food, respectively.

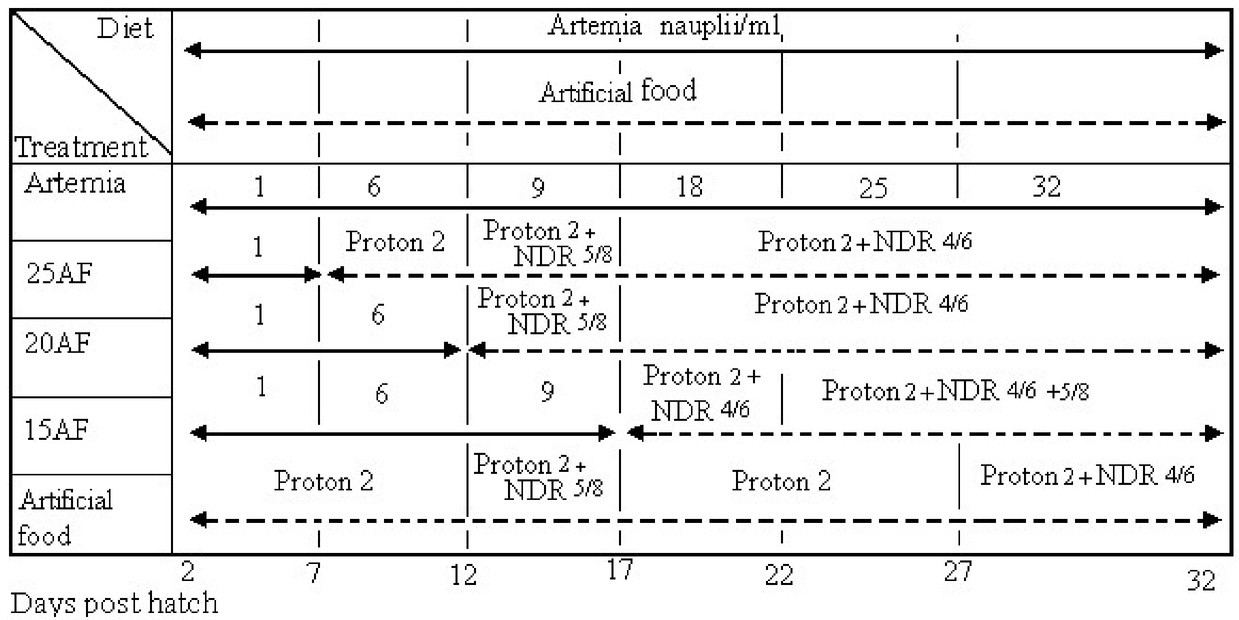
Time (dph)	*Artemia*	15AF	20AF	25AF	30AF
7	4.9 ± 1.0 ab	3.9 ± 0.9 c	4.4 ± 0.8 bc	5.1 ± 0.9 a	4.4 ± 1.1 bc
12	12.1 ± 3.2 a	9.8 ± 3.3 bc	11.7 ± 2.9 ab	9.7 ± 2.2 c	8.1 ± 2.3 c
17	26.2 ± 5.4 a	21.4 ± 5.0 b	18.0 ± 4.7 bc	17.5 ± 4.8 c	13.4 ± 4.2 d
22	44.8 ± 10.8 a	29.6 ± 11.7 b	29.6 ± 7.0 b	22.3 ± 8.1 c	15.0 ± 5.0 d
27	75.7 ± 24.8 a	41.3 ± 12.9 b	35.2 ± 9.0 bc	25.5 ± 10.4 cd	19.6 ± 7.1 d
32	104.5 ± 35.1 a	54.9 ± 21.2 b	39.8 ± 14.4 c	34.3 ± 15.1 c	20.2 ± 8.1 d

Table 4. Influence of diet on hepatocyte nuclear size (µm) of *Odontesthes argentinensis* larvae up to 32 dph. Data are expressed as mean ± SD. Thirty cells were analyzed per larva and two larvae were analyzed per treatment at each experimental time, except at 32 dph where four larvae per treatment were analyzed. Different letters indicate mean significantly different ($P<0.05$) between diets for the same experimental time. 15AF, 20AF, 25AF, and 30AF correspond to larvae fed for 15, 20, 25 and 30 days with artificial food, respectively. n.a. = non analyzed.

Time (dph)	*Artemia*	15AF	20AF	25AF	30AF
7	6.8 ± 0.3 a	n.a.	n.a.	n.a.	6.8 ± 1.1 a
12	8.7 ± 0.3 a	n.a	n.a	7.9 ± 0.6 a	8.3 ± 0.5 a
17	7.9 ± 0.6 b	10.1 ± 0.4 a	8.1 ± 0.1 ab	9.7 ± 0.4 ab	8.1 ± 0.8 b
22	7.9 ± 0.8 b	9.4 ± 0.1 ab	10.1 ± 0.1 a	8.2 ± 0.3 b	8.4 ± 0.4 ab
27	9.1 ± 0.5 a	10.9 ± 0.6 a	8.7 ± 1.0 a	9.4 ± 1.4 a	8.3 ± 0.2 a
32	8.1 ± 1.4 a	7.9 ± 0.2 a	8.2 ± 0.4 a	9.0 ± 0.7 a	7.3 ± 0.9 a

Table 5. Influence of diet on enterocyte length (μm) of *Odontesthes argentinensis* larvae up to 32 dph. Data are expressed as mean ± SD. Thirty cells were analyzed per larva and two larvae were analyzed per treatment at each experimental time, except at 32 dph where four larvae per treatment were analyzed. Different letters indicate mean significantly different ($P<0.05$) between diets for the same experimental time. 15AF, 20AF, 25AF, and 30AF correspond to larvae fed for 15, 20, 25 and 30 days with artificial food, respectively. n.a. = non analyzed.

Time (dph)	*Artemia*	15AF	20AF	25AF	30AF
7	11.6 ± 1.5 a	n.a.	n.a.	n.a.	14.2 ± 1.8 a
12	12.8 ± 0.3 a	n.a	n.a	15.7 ± 0.1 a	17.3 ± 1.9 a
17	15.2 ± 0.6 a	16.0 ± 3.6 a	15.6 ± 2.2 a	16.8 ± 0.1 a	13.6 ± 1.1 a
22	17.8 ± 4.3 a	16.8 ± 0.3 a	17.0 ± 1.3 a	16.8 ± 1.9 a	14.8 ± 0.2 a
27	18.6 ± 0.2 a	20.6 ± 0.4 a	20.5 ± 0.4 a	16.7 ± 2.2 a	19.5 ± 0.9 a
32	18.0 ± 2.1 a	18.1 ± 1.1 a	16.6 ± 1.7 ab	15.7 ± 1.3 ab	13.8 ± 1.3 b


Diet
Treatment
Artemia nauplii/ml
Artificial food
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25
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Proton 2
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Proton 2+NDR 4/6
20AF
1
6
Proton 2 + NDR 5/8
Proton 2+NDR 4/6
15AF
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Proton 2 + NDR 4/6
Proton 2+NDR 4/6 +5/8
Artificial food
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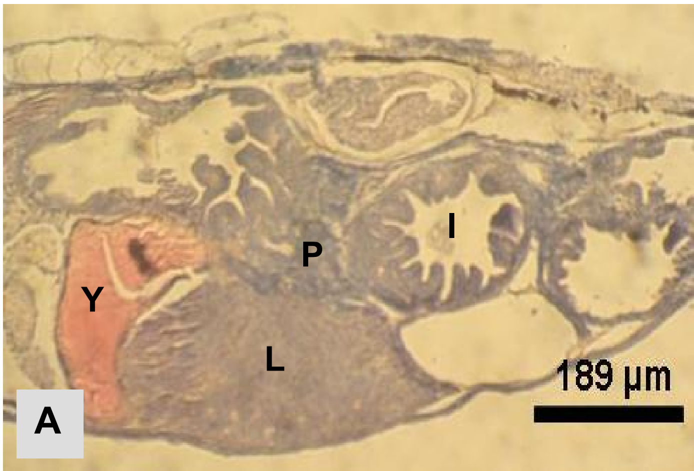
Days post hatch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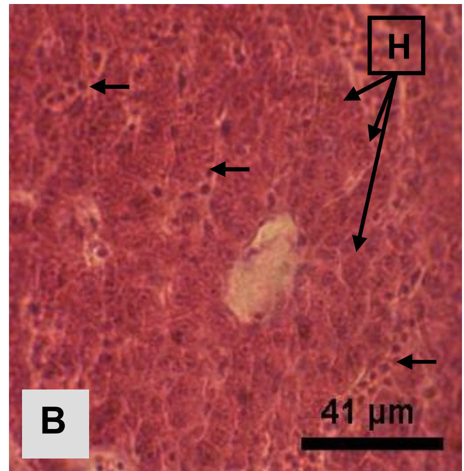
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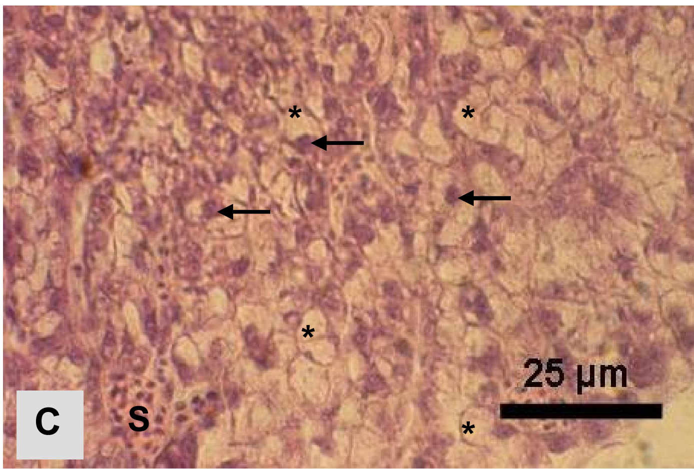
25 µm
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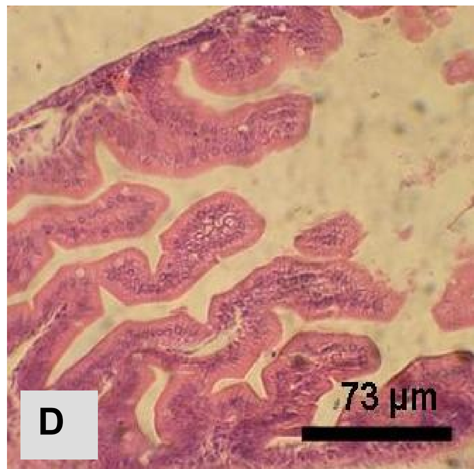
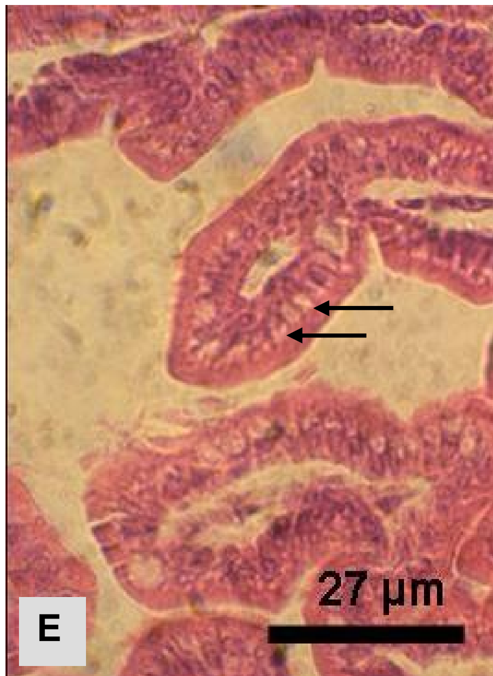
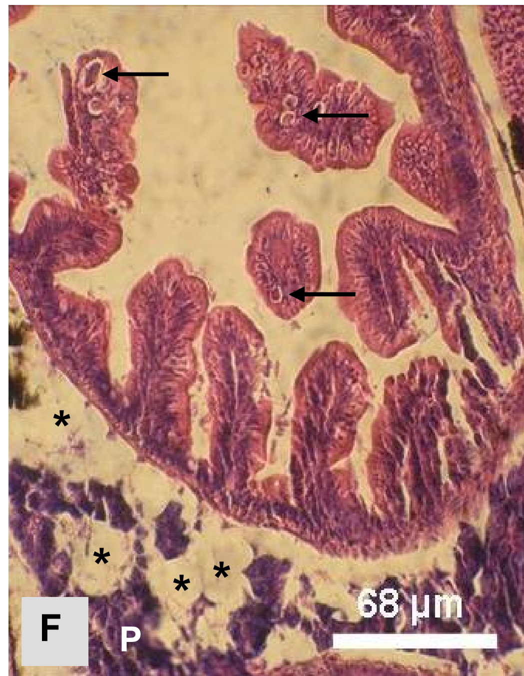
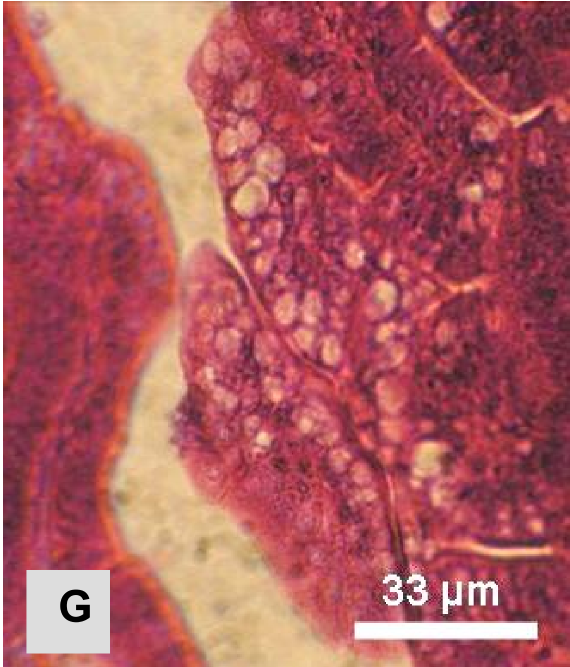
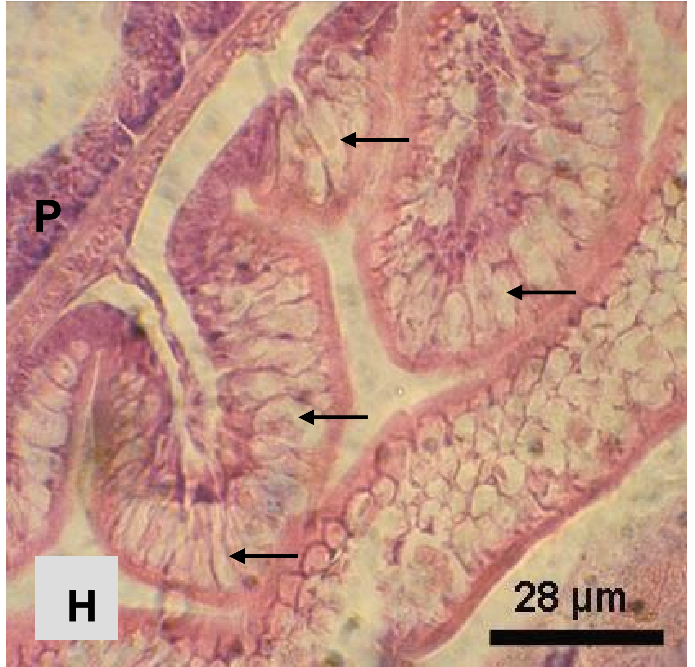
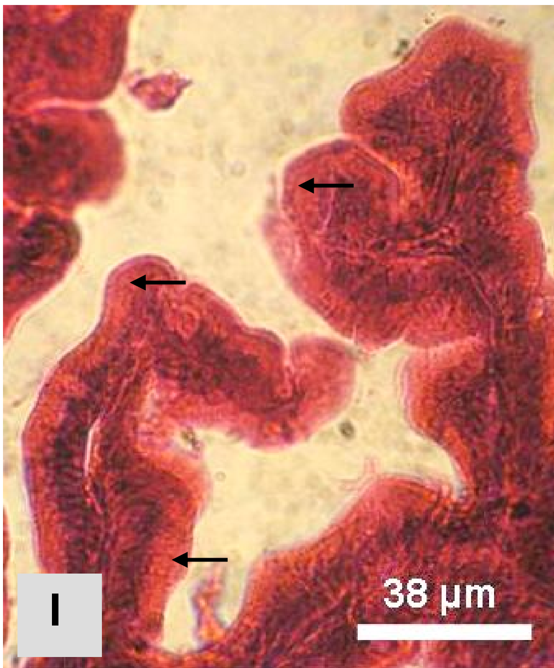
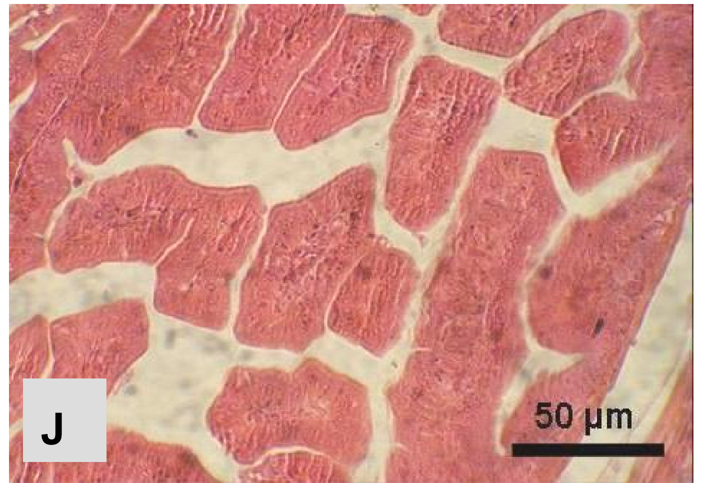
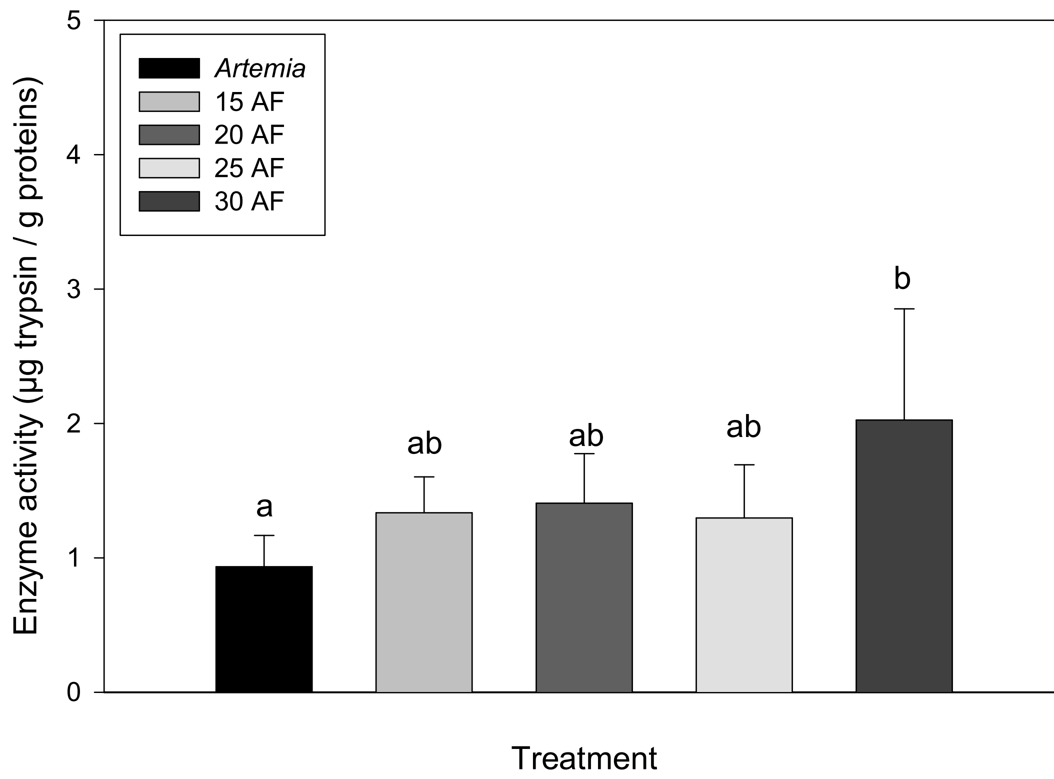

73 µm
D

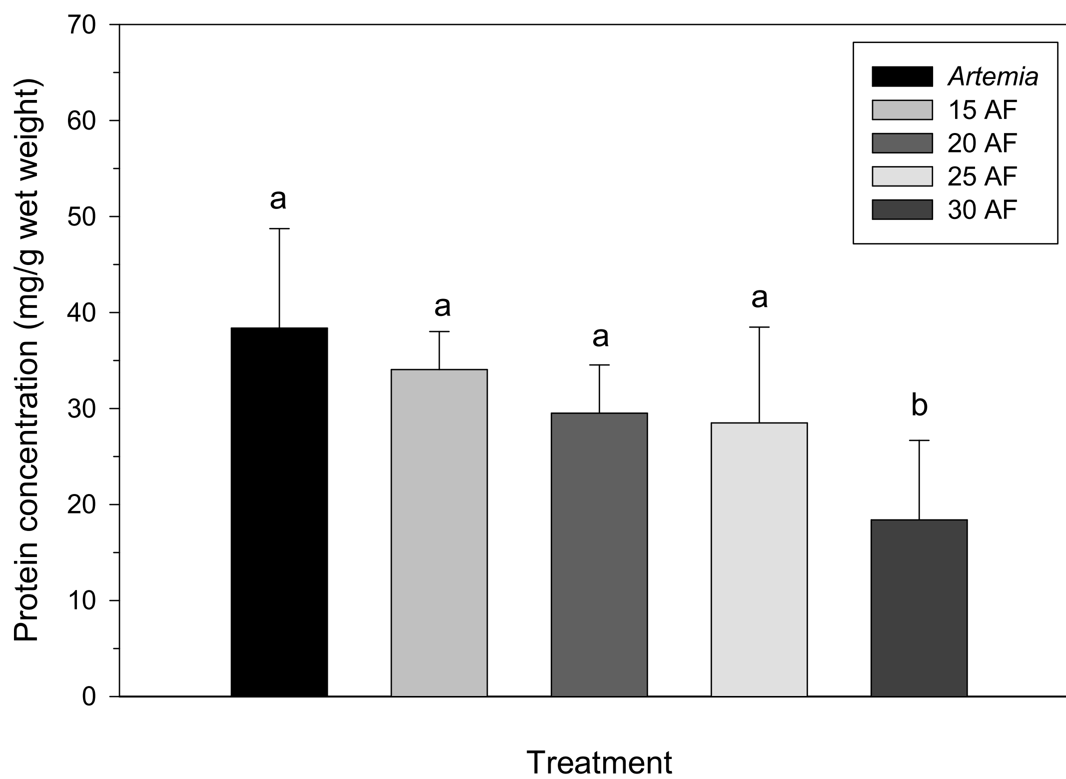

27 µm
E


68 µm
F
P


33 µm
G

P
28 µm
H

38 µm
I

50 µm
J



Enzyme activity (µg trypsin / g proteins)
5
4
3
2
1
0
Artemia
15 AF
20 AF
25 AF
30 AF
a
ab
ab
ab
b
Treatment



70
60
50
40
30
20
10
0
Protein concentration (mg/g wet weight)
Treatment
a
a
a
a
b
Artemia
15 AF
20 AF
25 AF
30 AF

V. CONCLUSÃO

Os dados de sobrevivência obtidos no presente estudo indicam que larvas (2 dias após a eclosão) do peixe-rei marinho *O. argentinensis* são capazes de se alimentar de ração como primeira alimentação. Além disso, estes dados mostram que uma dieta composta (náuplios de *Artemia* e ração) pode ser utilizada na seqüência de alimentação das larvas de peixe-rei, obtendo-se elevadas taxas de sobrevivência. Entretanto, os dados histológicos do trato digestório e da atividade corporal da tripsina claramente demontram que um possível retardo no desenvolvimento do trato alimentar reduziu a habilidade das larvas alimentadas com ração em digerir e acumular proteínas. Como conseqüência, foi observada uma redução considerável na taxa de crescimento destas larvas, e conseqüentemente da sua biomassa, após 30 dias de experimento (32 dias após a eclosão). No entanto, pesquisas adicionais são necessaries para avaliar os requerimentos nutricionais das larvas de peixe-rei, uma vez que um possível efeito da qualidade do alimento sobre os resultados obtidos no presente estudo não pode ser descartado. Assim, estudos futuros deveriam avaliar a digestibilidade e assimilação dos diferentes ingredientes presentes nas diferentes dietas empregadas no presente estudo para uma melhor seleção do momento adequado para se introduzir dietas microparticuladas no cultivo de larvas do peixe-rei *O. argentinensis*.

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