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Efeitos da suplementação alimentar com *n-3* HUFA na reprodução, larvicultura e desenvolvimento imunológico do barber goby, *Elacatinus figaro*.

MARCELO ROBERTO PEREIRA SHEI

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Orientador: Prof. Dr. Luis André Sampaio

Co-orientador: Prof. Dr. Luis Alberto Romano

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Dedicatória

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Sumário

RESUMO GERAL

O presente trabalho analisou o efeito da suplementação alimentar com ácidos graxos *n-3* HUFA sobre a performance reprodutiva e larvicultura do barber goby *Elacatinus figaro*, incluindo também a descrição da ontogenia do sistema imune. Durante quatro meses, dois grupos de reprodutores foram alimentados com ração comercial para peixes marinhos e biomassa de *Artemia* enriquecida e não enriquecida com *n-3* HUFA. Reprodutores alimentados com ambas dietas foram capazes de desovar continuamente, não demonstrando diferenças significativas para freqüência reprodutiva (12,6 ± 0,7 e 12,0 ± 1,2 dias) e o volume dos ovos (0,49 ±0,01 e 0,48 ±0,01 mm^3). A fecundidade foi maior no tratamento sem a suplementação de *n-3* HUFA (329 ± 36 e 517 ± 50 ovos / desova). Um segundo estudo avaliou o efeito da suplementação da dieta com *n-3* HUFA na sobrevivência e crescimento de larvas. Para isso, larvas recém eclodidas foram divididas em dois grupos experimentais, onde um grupo foi alimentado com rotíferos *Brachionus plicatilis* não enriquecidos e o outro grupo com rotíferos enriquecidos com *n-3* HUFA. Aos sete dias após a eclosão (dah), o crescimento foi maior no grupo alimentado com rotíferos enriquecidos, mas aos 14 dah não houve mais diferenças significativas entre os grupos (6.09 ± 0.62 and 5.69 ± 0.66 mm). Nesse momento, apesar de não haver diferenças significativas, a sobrevivência das larvas alimentadas com rotíferos enriquecidos com *n-3* HUFA foi três vezes maior (35,7 ± 3,1%) do que o grupo que recebeu rotíferos não enriquecidos (11,1 ± 5,2 %). Para a descrição do crescimento dos orgãos linfóides, amostras foram coletadas periodicamente entre 0 e 94 dah. A descrição se deu através de análises morfométricas do timo e rim e avaliação da expressão fenotípica (% / mm^2) de CD3 e CD4 nesses orgãos. No momento da eclosão foi possível observar a presença dos rins (0.31 ± 0,01 mm) e do timo (0.03 ± 0.03 mm), alcançando respectivamente 1.23 ± 0,15 mm e 90 ± 1.41 mm aos 94 DAH. Quanto ao CD3, a primeira expressão fenotípica foi constatada no timo no momento da eclosão (71,25 ± 8,42 % / mm^2) e no rim a partir dos 6 DAH (0,02 ± 0 % / mm^2), aos 94 DAH as expressões alcançaram 90 ± 1,41 e 53,67 ± 1,53 % / mm^2 no timo e rim respectivamente. O CD4 foi expresso inicialmente no timo aos 3 DAH (1,33 ± 1,15 % / mm^2) e no rim aos 12 DAH (5,33 ± 1,53 % / mm^2), alcançando respectivamente 22,00 ± 1,41 e 44,00 ± 2,00 % / mm^2 aos 94 DAH.

GENERAL ABSTRACT

The present study analyzed the effect of supplementation of *n-3* HUFA on reproductive performance and larviculture of barber goby *Elacatinus figaro*, also including the ontogeny of the immune system. During four months, two groups of breeders were fed commercial diets for marine fish and *Artemia* biomass enriched and not eniched with *n-3* HUFA. Broodstock feed with both diets were able to spawn continuously, showing no significant differences in spawning frequency (12.6 ± 0.7 and 12.0 ± 1.2 days) and egg volume (0.49 ± 0.01 and 0.48 ± 0.01 mm^3). Fecundity was higher in the treatment without supplementation of *n-3* HUFA (329 ± 36 and 517 ± 50 eggs / spawn). A second study evaluated the effect of dietary supplementation with *n-3* HUFA on larval survival and growth. Newly hatched larvae were divided into two experimental groups (200 larvae per group, with two replicates each) were one group was fed with non-enriched *Brachionus plicatilis* rotifers (NE) and the other group was fed with *n-3* HUFA enriched rotifers (ER). At 7 DAH, the growth was larger on the group fed with ER , and at 14 DAH there was no significant difference between groups (6.09 ± 0.62 and 5.69 ± 0.66 mm). However, the survival rate of larvae fed ER were three times higher (35.7 ± 3.1%) than the other group (11.1 ± 5.2%) who received NE. To describe the development of lymphoid organs, samples were collected periodically between 0 and 94 DAH. The description was given by morphometric analysis of the thymus and kidney and phenotypic expression (% / mm^2) of CD3 and CD4 in these organs. At 0 DAH was already possible to observe the kidney and thymus, reaching respectively 23.1 ± 0.15 mm and 90 ± 1.41 mm at 94 DAH. With regard to CD3, the first phenotypic expression was found in thymus at 0 DAH and kidney from 6 DAH, at 94 DAH expressions reached 90 ± 1.41 in the thymus and 53.67 ± 1.53% in the kidney. The CD4 was initially expressed in the thymus at 3 DAH and in kidney at 12 DAH reaching respectively 22.0 ± 1.41 and 44.0 ± 2% at 94 DAH.

INTRODUÇÃO GERAL

A pesca de peixes ornamentais marinhos teve início na década de 1930, mas somente a partir da década de 1980, suportada pelo desenvolvimento tecnológico, houve um grande aumento no mercado, tanto de organismos, como de insumos (Delbek, 2001). A atividade de pesca e transporte de peixes ornamentais ocorre em regiões tropicais e subtropicais, envoldendo cerca de 45 países (Wood, 2001). Em todo o mundo existem cerca de 1,5 a 2 milhões de aquários marinhos que sustentam a coleta de mais de 20 milhões de peixes marinhos de mais de 1.400 espécies anualmente (Wabnitiz et al., 2003).

Apesar do constante aumento da demanda, somente cerca de 70 espécies são produzidas por meio das técnicas de aquicultura, representando um volume inferior a 10 % do total de peixes comercializados (Moe, 2003; Wabnitiz et al., 2003; Molina e Segade 2011). Dentre as espécies produzidas, a grande maioria é de reprodução monogâmica, ovoposição de ovos demersais e cuidado parental (Wabnitiz *et al*., 2003).

O limitado número de espécies criadas em cativeiro e os problemas relacionados ao gerenciamento pesqueiro das áreas e espécies capturadas caracteriza a atividade como extrativista, sendo em muitos casos desordenada (Tlusty, 2002; Tissot et al., 2010). Por isso, a preocupação das agências ambientais em minimizar os impactos sobre as espécies recifais pode ser minimizada sem sacrificar o mercado através do desenvolvimento de protocolos de produção das espécies mais exploradas (Pomeroy et al., 2006; Olivotto et al., 2011).

Como ocorrido com outras espécies de peixes marinhos, o desenvolvimento de protocolos para a produção de formas jovens de espécies ornamentais apresentam problemas relacionados ao processo produtivo. As técnicas e informações sobre indução à ovulação e desova, o desenvolvimento nas idades iniciais, a primeira

alimentação e a mudança do alimento vivo para dietas inertes são pouco conhecidos (Ostrowski e Laidley, 2001; Calado, 2006; Moorhead e Zeng, 2010).

A Família Gobiidae apresenta o maior número de espécies entre os peixes marinhos (Nelson, 1994). Ela também tem boa representatividade no mercado internacional de peixes ornamentais marinhos, representando entre 5 a 7 % das vendas (Wabnitiz *et al*, 2003). Características da família podem proporcionar a obtenção de desovas naturais frequentes, sendo que o grande tamanho dos ovos e o pequeno tamanho dos adultos são características que facilitam a sua criação em cativeiro (Colin, 1975; Olivotto *et al.*, 2005; Shei et al., 2010).

Os Gobiidae do gênero *Elacatinus* são peixes recifais, tipicamente coloridos e brilhantes e envolvidos com relação simbiôntica de limpeza com peixes maiores (Colin, 1975). O grupo é representado por cinco espécies no oceano Pacífico, 13 no oeste do oceano Atlântico norte e três no oeste do Atlântico sul (Guimarães *et al*., 2004). Esses animais possuem como característica o hábito de se alimentar de ectoparasitas de outros peixes, destacando sua importância ecológica, além da importancia comercial como espécie ornamental (Valenti, 1972; Gasparini *et al.*, 2004).

Quanto à estratégia reprodutiva, espécies de *Elacatinus* possuem hábito monogâmico (Harding *et al*., 2003; Whiteman e Côté, 2003) com alta freqüência reprodutiva e fecundidade variando de 250 a 1.000 ovos por desova (Olivotto *et al*., 2005; Meirelles *et al*., 2009; Shei et al., 2010). O desenvolvimento ontogênico dessa espécie é caracterizado por larvas planctônicas que ao completarem a metamorfose passam a ter comportamento bentônico (Olivotto *et al.*, 2005; Meirelles *et al*., 2009; Shei et al., 2010).

Figura 1: Exemplar adulto do barber goby *Elacatinus figaro*. Foto: Antônio Amaral.

O barber goby *Elacatinus figaro* é endêmico da costa brasileira, apresentando ampla distribuição, ocorrendo em recifes e costões rochosos do litoral da Paraíba até Santa Catarina (Sazima *et al*., 1996). É um peixe de pequeno porte, que atinge aproximadamente 4 cm de comprimento, de coloração amarela bastante viva e uma listra preta que segue da cabeça até a cauda (Sazima *et al*., 1996). Foi uma das espécies mais importantes no comércio de peixes ornamentais marinhos do país até ser incluída na lista de espécies em extinção devido à sobrepesca (IBAMA, 2004; Gasparini *et al*, 2004).

Como uma espécie nova na aquicultura, apenas informações iniciais relacionadas ao comportamento reprodutivo, desenvolvimento embrionário e larvicultura foram relatadas até o presente momento (Meirelles et al., 2009; Shei et al., 2010, 2012; Côrtes & Tsuzuki, 2011)

Os peixes marinhos geralmente têm pouca ou nenhuma capacidade de sintetizar os ácidos graxos altamente insaturados (*n-3* HUFA) "de novo". Isto é atribuído à ausência da enzima Δ-5 desaturase (Sargent 1995; Sargent *et al*., 1997; Corraze, 2001), consequentemente, o ácido eicosapentaenóico (EPA, 20:5 *n-3*) e o ácido docosahexaenóico (DHA, 22:6 *n-3*) são considerados ácidos graxos essenciais (EFA)

nas dietas da grande maioria dos peixes marinhos (Kanazawa, 1997; Sargent *et al.*, 1999a; 2002).

Os ácidos graxos essenciais (EFA) são importantes por serem os maiores componentes dos fosfolipídios das membranas celulares, incluindo as do sistema nervoso, atuando também no transporte de lipoproteínas (Sargent, 1995; Corraze, 2001; Sargent *et al.*, 2002).

Com respeito às larvas de peixes marinhos, estudos com EFA têm demonstrado que esses nutrientes possuem grande importância no desenvolvimento larval, uma vez que a sua deficiência pode afetar o desenvolvimento normal dos sistemas visual e nervoso central (Sargent *et al.,* 1999b). Além disso, sua falta também afeta a taxa de crescimento, tolerância ao estresse, sobrevivência, nível de pigmentação (Copeman *et al*., 2002; Vagelli, 2004; Olivotto *et al.*, 2008), capacidade de natação e alimentação (Benítez-Santana *et al*., 2007).

O estado nutricional dos reprodutores em períodos de desova pode afetar diretamente a qualidade dos seus descendentes (Lavens *et al.*, 1999; Yanes-Roca *et al*., 2009). O acumulo de nutrientes essenciais como ácidos graxos e vitaminas nos ovos depende das reservas da fêmea e, consequentemente, da dieta no período que antecede a maturação dos ovócitos (Bell *et al.*, 1997). Dietas deficientes em EFA afetam diretamente o desenvolvimento gonadal, diminuindo a fecundidade e a qualidade de ovos e larvas (Rodrigues *et al.*, 1998; Corraze, 2001), especialmente em espécies de desovas constínuas, que possuem curtos períodos vitelogênicos (Izquierdo *et al.*, 2001). Estudos com espécies marinhas de desova parcelada demonstram que reprodutores alimentados com quantidades apropriadas de *n-3* HUFA durante o período de maturação gonadal, produziram ovos de melhor qualidade, obtendo maiores taxas de eclosão (Fernández-Palacios *et al.*, 1998; Yanes-Roca *et al*., 2009).

Dentre os alimentos vivos empregados tradicionalmente na aqüicultura, destacam-se os rotíferos e a *Artemia,* dois dos principais organismos-alimento usados para larvas de peixes marinhos. Sabe-se que é possível incorporar quantidades razoáveis de EFA nos rotíferos utilizando-se microalgas, mas que quantidades superiores desses EFA são alcançadas com a utilização de emulsões comerciais (Lavens e Sorgeloos, 1996; Roo *et al*., 2008). No entanto, também é conhecido que a *Artemia* é deficiente em nutrientes essenciais, especialmente em ácidos graxos poliinsaturados de cadeia longa como o DHA e o EPA (Han *et al*., 2000; Holt, 2003). A deficiência de ácidos graxos essenciais é uma das razões para que a *Artemia* não seja um alimento completo para o crescimento de larvas de peixes marinhos (Watanabe *et al.,*1983; Sargent *et al.*, 2002).

Ácidos graxos são precursores de compostos biológicos altamente ativos, os eicosanóides, que englobam as prostaglandinas, tromboxanos e prostaciclinas (Corraze, 2001). Os eicosanóides são autócrinos como hormônios, produzidos pelas células possuindo ação imediata e de forma local. Possuem interações diretas em processos fisiológicos, como a coagulação sanguínea, processo inflamatório, reprodução e resposta imune (Izquierdo, 1996; Calder, 1999; Sargent *et al.*, 2002; Puangkaew *et al*, 2004; Glencross, 2009).

O sistema imune dos peixes em termos gerais é semelhante ao dos vertebrados superiores (Flajnik, 1996). A resposta imune dos vertebrados, incluindo os peixes pode ser dividida em dois tipos: a resposta imune inespecífica e a resposta combinada ou específica. A primeira é caracterizada por ser antígeno independente, a qual apresenta uma resposta similar nas possíveis presenças futuras de um mesmo agente infeccioso por não haver memória imunológica. Este sistema atua como uma primeira defesa na presença de um agente patógeno, podendo controlar diversas infecções sem a

dependência do sistema específico (Flajnik, 1996; Levraud e Boudino, 2009; Romano, 2010, Klosterhoff e Romano, 2012). O segundo tipo é induzível e exige a presença de células que reajam especificamente com o antígeno indutor, os linfócitos. A denominação de resposta combinada se deve pelo envolvimento de dois elementos: a resposta humoral, mediada por anticorpos e a resposta celular, controlada principalmente, por linfócitos T (Bernstein *et al*., 1998; Kourilsky *et al*., 1998; Levraud e Boudino, 2009).

O rim anterior e o timo são os maiores orgãos linfóides nos teleósteos. Nas espécies dulcícolas, apesar do rim poder ser o primeiro orgão hematopoiético, o timo é o primeiro a se tornar linfóide (Lam et al., 2004). Quanto as espécies marinhas, a ordem em que os principais orgãos linfóides se desenvolvem é dada pelo rim, baço e finalmente, o timo, No entanto, nas fases larvais, o baço possui uma função mais eritropoiética do que linfóide (Schroder et al, 1998; Zapata et al., 2006).

Devido ao fato das doenças serem responsáveis por grandes perdas econômicas na produção de pescado em todo o mundo, o estudo dos mecanismos de defesa são importantes para o sucesso da aquicultura. O conhecimento da ontogenia do sistema imune permite a determinação do momento em que o organismo estaria apto para receber vacinas e enfrentar situações estressantes, como o ocorrido no manejo de transporte e período de engorda (Kato et al., 2004). Informações relacionadas a ontogenia do sistema imune de peixes são restritas e ainda mais reduzidas em espécies marinhas, sendo conhecidas para espécies como o robalo europeu, *Dicentrarchus labrax*, o halibute do Atlântico, *Hipoglossus hipoglossus* e o bacalhau do Atlântico, *Gadus morhua* (Padrós & Crespo, 1996; Zapata et al., 2006).

Os altos custos de operação para o desenvolvimento de pesquisas com reprodutores (Izquierdo *et al.*, 2001), o pequeno número de espécies estudadas e as

diferenças dos órgãos linfóides nos peixes marinhos e dulcícolas destacam a necessidade de uma espécie marinha que possa ser utilizada como modelo experimental (Schroder et al, 1998; Grunwald and Eisen, 2002; Zapata et al., 2006). Sendo assim, o tamanho reduzido das espécies de peixes ornamentais pode ser interessante para o desenvolvimento dos estudos sobre reprodução em peixes

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OBJETIVOS

Objetivo geral

O presente trabalho teve como objetivo estudar o efeito do enriquecimento da dieta de reprodutores e larvas com *n-3* HUFA e descrever a formação dos órgãos linfóides e o desenvolvimento do sistema imune do barber goby *E. figaro*

Objetivos específicos

1. Analisar o efeito do uso de alimentos ricos em ácidos graxos insaturados na dieta de reprodutores do barber goby na fecundidade e freqüência reprodutiva.

2. Comparar a taxa de crescimento e o percentual de sobrevivência das larvas do barber goby alimentadas com rotíferos enriquecidos com ácidos graxos insaturados desde a primeira alimentação.
3. Descrever a formação dos órgãos linfóides e o desenvolvimento ontogenético do sistema imunológico de larvas e juvenis do barber goby.

Capítulo 01

Supplemention *of n-3* HUFA in broodstock diet on spawning frequency, fecundity and egg volume of barber goby, *Elacatinus Figaro*

Abstract.

We studied the supplementation of *n-3* HUFA in broodstock diet on spawning frequency, fecundity and egg volume of barber goby. During four months, two broodstock groups were feed with a commercial diet for marine fish (16 % lipids, 59 % crude protein) and *n-3* HUFA enriched and non-enriched *Artemia* biomass. Independently of supplemental *n-3* HUFA, both diets were able to condition the breeders to spawn continuously during the studied period, showing no significant differences for spawning frequency (12.6 ± 0.7 and 12 ± 1.2 days) and egg volume (0.49 ±0.01 and 0.48 ±0.01 mm^3) among the treatments. Fecundity was highest on group feed with no *n-3* HUFA supplementation (329 ± 36 and 517 ± 50 eggs per spawn). Thus, it may be assumed that the amount of lipids and the fatty acid profile contained in the commercial feed supplied is sufficient to condition the barber goby to spawning, and the supplementation offered by *n-3* HUFA enriched *Artemia* biomass exceeded the optimal levels, affecting negatively the reproductive performance. Therefore, it is suggested that studies dosing different amounts of *n-3* HUFA in the diet of broodstock be developed in order to optimize the reproductive management of this species.

Key words: Yellow line goby, neon goby, marine ornamental, *n-3* enrichment, reproduction, feeding, nutrition.

Introduction

Teleost female require a large amount of macro and micronutrients during the maturation process. This involves a considerable mobilization of stored materials and diversion of dietary nutrients into gonad development (Bell et al., 1997; Bruce et al., 1999). The levels of lipids and fatty acid composition have been correlated with reproductive parameters of many species of fish, including egg quality, spawning frequency, fertilization and hatching rates, and larval quality (Rainuzzo et al., 1997; Yanes-Roca et al, 2009).

Marine fish generally have limited or no ability to synthesize highly unsaturated fatty acids (*n-3* HUFA) "de novo". This is attributed to the absence of the enzyme Δ-5 desaturase (Sargent 1995, Sargent et al., 1997; Corraze, 2001), consequently, eicosapentaenoic acid (EPA, 20:5 n-3) and docosahexaenoic acid (DHA, 22: 6 n-3) are considered essential fatty acids (EFA) in the diets of the majority of marine fish (Kanazawa, 1997, Sargent et al., 2002). Deficient diets in EFA affect the gonadal development, reducing fertility and quality of eggs and larvae (Rodrigues et al., 1998; Corraze, 2001), especially in species with short vitellogenic periods (Izquierdo et al., 2001).

The market for ornamental species for the aquarium trade has become a multibillion-dollar global business, with the participation of millions of specimens of fish and invertebrates being traded. Despite the constant increase in demand, less than 10% of the marine fish species marketed are produced by aquaculture (Wabnitiz et al., 2003; Molina & Segade 2011). Thus, the concern of environmental agencies to minimize the impact on coral reef fish species, can be reduced without sacrificing the market, through the development of production protocols for the most heavily harvested species (Pomeroy et al., 2006; Olivotto et al., 2011). Among the requirements necessary for successful mass production of juvenile fish, especially for new species, is the control of reproduction performance (Izquierdo et al., 2001; Ostrowski & Laidley, 2001, Olivotto et al., 2003).

The barber goby *Elacatinus figaro* is an endemic species to the Brazilian coast and is of great interest for the international marine ornamental market due to its small size (<4 cm), bright colours and cleaning activity (Colin, 1975, Sazima et al., 1997; Gasparini et al., 2005). As a new aquaculture species, only initial information related to reproductive behavior, embryo development and larviculture has been studied (Shei et al., 2010, 2012; Côrtes & Tsuzuki, 2011). Thus, the objective of the present work was

to evaluate the influence of a commercial diet supplemented with *Artemia* biomass enriched with *n-3* HUFA on barber goby, *Elacatinus figaro* spawning frequency, fecundity and egg size.

Materials and methods.

Broodstock husbandry

Adult specimens of *E. figaro* were collected in the Baia de Todos os Santos - Salvador- BA, Brazil using nets and scuba diving (authorization SISBIO 22859-1). The animals were air-shipped in sealed bags to the Laboratory of Estuarine and Marine Fish Culture of the Universidade Federal do Rio Grande - FURG. After acclimatization, fish were placed in 30 L (40 x 30 x 30 cm) aquariums connected to a RAS consisting of a main pump (12 m^3 / h), an HSA skimmer (Plaspiral – Brazil), biological trickling filter (40 m^2 surface), ultra-violet sterilizer 55 W (> 30,000 μS / cm^2 / s, Sibrape – Brazil), three 250 W heaters with thermostat (Visitherm – Italy) and a 200 μm bag filter.

The aquariums contained four 32 x 40 mm PVC fittings for shelter. They were illuminated by fluorescent bulbs (6,500 k) and the photoperiod was set at 12L: 12D, the temperature ranged from 25 to 27 °C, salinity was 32‰, dissolved oxygen > 6 mg/L (YSI, 55A), pH at 8.0 - 8.4 (YSI, pH 100 - EUA) and NH_3 (UNESCO, 1983) and NO_2 (Bendschneider & Robinson, 1952) <0.02 mg/ L.

Mated pairs formation

Groups of 5 to 6 fishes were randomly added to each aquarium in order to obtain the desired pairs. Once a couple was formed, which was defined as the acceptance of one fish by another, or the observation of the actual courtship behavior, they were isolated and considered as a mated pair. Animals that were constantly defending territory or repelled were rotated among the tanks until the formation of 10 couples. Each pair was kept in isolated tanks containing a PVC pipe (32 mm in diameter and 80 mm length) with an internal plastic film as a suitable substrate for egg laying.

Five matted pairs were assigned to each of two experimental diet treatments. During four months, all 10 matted pairs were fed until apparent satiation four times a day: twice (8:00 am and 4:00 pm) with commercial dry feed (INVE, NRD, 500 – 800 μm; 59 % crude protein and 16 % lipids) and twice (12 pm and 7:30 pm) with no enriched *Artemia* nauplii biomass (NE) or *n-3* HUFA enriched *Artemia* metanauplii biomass (EA).

Aquariums were checked daily for spawning before each feeding routine. The first two spawning of each pair were discarded in order to exclude the effect of previous diet. Thereafter, the reproductive performance of each pair was evaluated by the spawning frequency and by counting and measuring the eggs (n=10, length and width) under a stereomicroscope equipped with a micrometric eyepiece (Wild, M5A). The volume of eggs, as well used in clown fish (Green and McCormick, 2005) was estimated using the formula for a cylinder: volume= $\pi r^2 L$, where r is egg radius and L is egg length.

Zooplancton culture

Artemia nauplii and enriched metanauplii used to feed the broodstock were obtained by hatching *Artemia* cysts (EG, Inve - USA) in 20 L conical tanks. For the *n-3* HUFA enrichment, the nauplii were conditioned for 24 hours with a commercial enrichment emulsion (Easy DC SELCO - Belgium). The protocols used were provided by the manufacturer. After this, the *Artemia* biomass for both treatments was frozen and offered to broodstock. This process was repeated every two weeks.

Statistical analysis

The spawning frequency and fecundity were analyzed using Student t test and egg volume were analyzed with Kruskal-Wallis using Statistic 7.0 Software®. A probability of 0.05 was considered to determinate statistical difference between the means. Results are presented as the means ± standard error of the data.

Results

The t-test found no significant differences (P = 0.67) for spawning frequency between the fish fed both diets. The average interval of spawning was 12.6 ± 0.7 and 12.0 ± 1.2 days for broodstock fed EA and NE diet, respectively.

The fecundity was significantly affected by the broodstock diet (P = 0.004). Broodstock fed on EA diet produced a lower number of eggs than those who received NE diet (Figure 1), with a respective mean of 329 ± 36 and 517 ± 50 eggs per spawn.

Independently of the diet of broodstock, egg volume was not significantly affected (P=0,47). Average egg volume was 0.49 ±0.01 and 0.48 ±0.01 mm^3 for the breeders who received EA and NE diet, respectively.

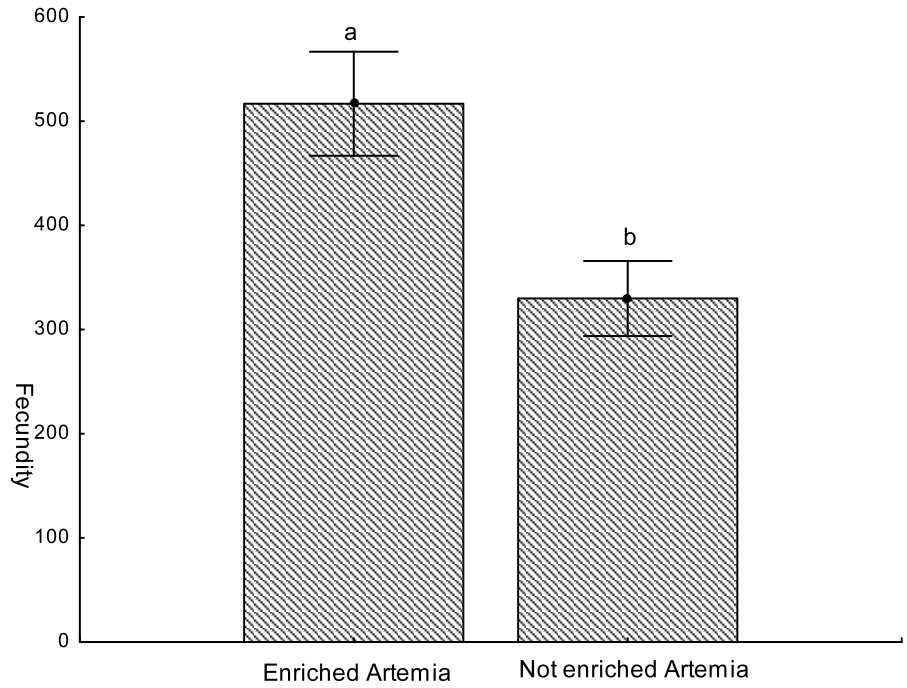

Figure 1. Broodstock fecundity rate between diets during four months. Values are expressed as means ± standard error. Different letters indicate significant differences (P<0.05).

Discussion

Nutrition of broodstock fish is one of the least known and studied themes in aquaculture, this is due in large part, by the need for large structures for the maintenance of adult animals during extended trials, and consequently high costs of operation (Izquierdo et al., 2001). This difficulty highlights the need for a marine species that can be used as an experimental model, as the zebrafish *Danio rerio* (Grunwald and Eisen, 2002; Callan et al., 2011).

The effect of dietary *n-3* HUFA on reproductive performance has been studied in several fish species of commercial interest (Bruce et al., 1999; Ling et al., 2006; Furuita et al., 2007; Zakeri et al., 2011). Most of these studies related the dietary *n-3* HUFA deficiency with lower quality of reproductive performance, egg and larval quality and chemical composition while the effect of dietary *n-3* HUFA levels on egg production differed among species (Furuita et al., 2002; 2007; Callan et al., 2011). Apart from dietary EFA deficiencies causing detrimental effects in fish, their excess

has been also reported to have a negative effect on reproductive performance of fish (Izquierdo et al., 2001, Furuita et al., 2002).

In the present study, independently of supplemental *n-3* HUFA, both diets were able to condition the breeders to spawn continuously during 4 months, with no significant differences on spawning frequency and egg volume. However, the supplementation with *n-3* HUFA resulted in a significantly lower fecundity. Similar results were also described with gilthead sea bream broodstock by Fernandez-Palacios et al. (1995), where fish fed a diet containing high amounts of *n-3* HUFA (3.5%) showed lower fecundity. Thus, it may be assumed that the amount of lipids (16%) and the fatty acid profile contained in the commercial feed and *Artemia* nauplii supplied is sufficient to condition the barber goby to spawning, and the supplementation offered by *n-3* HUFA enriched *Artemia* biomass exceeded the optimal levels for the species. High dietary *n*-3 HUFA levels could affect the brain–pituitary–gonad endocrine axis since both EPA and DHA have been found to reduce in vitro the steroidogenic action of gonadotropin in the ovary of teleost fish (Mercure and Van Der Kraak, 1995; Izquierdo et al, 2001; Sorbera et al, 2001).

By the characteristic of the small number and demersal eggs, the egg volume becomes an important tool to characterize reproductive data and quality of eggs. In fishes, embryo and larval characteristics such as egg size, growth rate and viability are affected by the body condition and genotype of the female parent (Kerrigan 1997, Marteinsdottir and Steinarsson 1998, Green and McCormick, 2005). Due to the fact the animals were obtained from the nature, it is not possible to determine the age and other conditions that affect the reproductive performance.

It is suggested that a scientific and rigorous approach is needed in order to develop and optimize the breeding protocols and successfully increase the number of marine ornamental species reared (Olivotto et al., 2003; 2011).

Conclusions

It is possible to condition barber goby broodstock to spawn using only commercial feed. The possible excess of *n-3* HUFA in the broodstock diet did not affect the spawning frequency and egg size, but the fecundity decreased with the dietary *n-3* supplementation. Thus, it is suggested that studies dosing different amounts of *n-3* HUFA in the diet of broodstock be developed in order to optimize the reproductive management of this species.

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Capítulo 2

Use of commercial live feeds enrichment during first feeding period of the barber goby *Elacatinus figaro*.

Abstract. The first feeding period is the most critical phase for the production of marine fish larvae. The utilization of *n-3* HUFA enrichment on live feed has improved the results for several species during the larviculture. To evaluate the effect of *n-3* HUFA enrichment on survival and growth of the barber goby *Elacatinus figaro* Sazima, Moura & Rosa, 1997, newly hatched larvae were divided in two experimental groups (200 larvae per group, with two replicates each). One group was fed on non-enriched rotifers *Brachionus plicatilis* and the other group was fed with n-3 HUFA enriched rotifers. After 14 days of experiment, survival of larvae fed *n-3* HUFA enriched rotifers was three times higher (35.7 ± 3.1%) than those fed non-enriched rotifers (11.1 ± 5.2 %), however this difference was not significant. Growth was faster for larvae fed *n-3* HUFA enriched rotifers after the first week of life, but at the end of 14 days, it was no longer significantly different between the two groups (6.09 ± 0.62 and 5.69 ± 0.66 mm). The results of this experiment suggest that barber goby should be fed n-3 HUFA enriched rotifer in order to maximize juvenile production.

Key words: marine ornamentals, neon goby, larviculture.

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Introduction. Following the world technological and economical development in the last decades, the ornamental fish market is well established and globalized. Home aquariums have made more popular and accessible to common households, increasing the annual consumption of dry goods and live organisms to a multi billion dollars market (Wabnitiz et al, 2003; Calado, 2006).

Similar to the market of marine table fish, where the technology for aquaculture production is available for a small number of species, production of ornamental species

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is also limited. However, this is a market based on species diversity, commercialized by units instead of weight, and it cannot be supplied with the large production of a few species, as it is a diversified market (Wabnitiz et al, 2003; Pomeroy et al, 2006). Therefore, it is necessary to acquire technology for the production of several species of ornamental marine fish.[2]

The development of protocols for cultivation of ornamental fish faces several problems related to the production process, which resemble the difficulties found in the production of juvenile forms of other species of marine fish. The small body size and low fecundity of marine ornamental fish species are two of the main problems fish producers have to cope with (Ostrowski and Laidley, 2001; Holt, 2003; Moorhead and Zeng, 2010).

Most marine fish larvae species are produced using live feed during the first stages of development. Rotifers, *Brachionus spp*. and *Artemia* nauplii are the most common preys used, but despite the advantages on easy production of large quantities and high densities, it is known that these organisms do not have the fatty acid profile required by most marine fish larvae (Sargent et al, 1999). In order to improve the output of larviculture, studies using enrichment of live feeds with *n-3* HUFA have shown positive results during this period of cultivation (Vagelli, 2004; Olivotto et al, 2006; Drillet et al, 2011).

Several studies on the requirement of *n-3* HUFA have shown that these nutrients are essential for marine fish larvae. Its deficiency can result in a poor development of the visual and central nervous systems (Sargent et al., 1999). It also negatively affects the growth rate, stress tolerance, pigmentation, and ultimately survival (Copeman et al. 2002; Olivotto et al., 2008).

The Family Gobiidae has the largest number of species among marine fish (Nelson, 1994). Gobiidae have a good representation in the international market of marine ornamental fish, accounting for 5 to 7% of total sales (Wabnitiz *et al*, 2003, Olivotto et al, 2005). The barber goby *Elacatinus figaro* is an endemic species from the Brazilian coast with high demand in the international marine ornamental trade market (Gasparini et al, 2005). However, since 2004 it is listed as an endangered species by the Brazilian Institute of Environment and Natural Resources and can no longer be legally collected from the wild (IBAMA, 2004).

Initial information related to the reproduction, embryo development and larviculture of the barber goby has been described recently (Meireles *et al*, 2009; Shei *et al*, 2010), and high mortality rates were reported during the first feeding period (Shei *et al*, 2010). Expecting to contribute with technology for aquaculture production of

juvenile barber gobies this work aims to test the effect of *n-3* HUFA enrichment of rotifer on growth and survival rate of barber goby larvae during the first feeding phase.

Materials and Methods

Broodstock

Adult barber gobies were collected (permission SISBIO 22859-1) by scuba diving in the Baía de Todos os Santos, coast of Bahia state – Brazil, and transported to the Laboratory of Marine Fish Culture of the Federal University of Rio Grande - FURG.

Barber gobies pairs were kept in five tanks (30 L) connected to a recirculating aquaculture system (RAS) composed of a 50 μ bag filter (OceanTech, Brazil), three 250 W heaters (Marineland, Italy), a protein skimmer (Plaspiral 1000 HSA, Brazil), a trickling biofilter (7 m^2 total surface), a pressurized mechanical filter (30 μ – Acquafiltros, Brazil) and a 55 W UV sterilizer (> 30.000 μW / cm^2 / s, Sibrape, Brazil). All tanks were supplied with a PVC pipe (32 mm diameter and 80 mm length) with an internal plastic film as suitable substrate for egg laying. Photoperiod was maintained at 12 h light / 12 h dark. Temperature and dissolved oxygen (YSI, 55A, USA), and salinity (Atago salinity refractometer, Japan) were measured daily, while pH (YSI, pH 100, USA), ammonia (UNESCO, 1983) and nitrite (Bendschneider & Robinson, 1952) methods were measured weekly.

Fishes were fed four times per day, twice (8:00 am and 4:00 pm) with commercial dry feed (59 % crude protein, and 16 % lipids, INVE, NRD, Belgium) and twice (12 am and 7:30 pm) with frozen *n-3* HUFA enriched *Artemia* metanauplii biomass. Under these conditions, fishes spawned every 9 – 10 days and the embryo development lasted 7 days.

Zooplankton culture

n-3 HUFA enriched *Artemia* metanauplii used to feed broodstock were obtained by hatching *Artemia* cysts (EG, Inve, USA) in 20 L conical tanks. For the DHA enrichment, the nauplii were conditioned for 24 hours with commercial enrichment emulsion (Easy DHA SELCO, Belgium), following the protocol suggested by the manufacturer. *n-3* HUFA enriched *Artemia* metanauplii biomass was frozen and offered to broodstock. This process was repeated every two weeks.

Rotifers (*Brachionus plicatilis*) offered to the larvae were cultured on 150 L tanks on *Nannochloropsis oculata* and commercial dry feed (S.parkle – INVE Belgium), at salinity 30 and 25 °C temperature. *n-3* HUFA enriched rotifers were obtained with commercial formulations (Algamac 2000– Aquafauna Bio-Marine, Inc, USA) following the protocol provided by the manufacturer.

Larval rearing

Two hours before larvae began to hatch, the plastic film with adhered eggs was removed from the PVC pipe and transferred to a beaker (1 L) with gentle aeration until all larvae hatched. Larvae were than counted, measured under a stereomicroscope (WILD, M5A, Germany) and split into four tanks. Each tank was stocked with 200 larvae. Group A fed with non-enriched rotifers and group B fed with enriched rotifers. In both groups rotifers were kept at density of 10 ind / mL, they were replaced 4 times / day in order to assure the quality of the prey. Tanks were maintained with green water (*Nannochloropsis oculata*, 500.000 cells/mL) during the experimental period. Both experimental groups were kept under extended photoperiod 24 L: 0 D.

Larvae were reared in four 90 L cylindrical tanks (black wall and white bottom) connected to a RAS composed by bag filter (50 μ) to retain rotifers from the tanks, two 250 W heaters, a protein skimmer (Plaspiral 800R, Brazil), a fluidized biofilter (bioballs, 5 m^2 total surface) and a 30 W UV sterilizer (> 30.000 μW / cm^2 / s, Plaspiral, Brazil). Larvae from all tanks were measured at 0, 7 and 14 days after hatching (dah). Survival was estimated by counting all remaining larvae at the end of the experiment 14 days after hatch.

Statistical analysis

The results were analysed using Student T test, with Statistic 7.0 Software®. A probability of 0.05 was considered to determinate statistical difference between the means. Results are presented as the means ± standard deviation of the data.

Results and Discussion

Water quality – larval rearing and broodstock.

During the experimental period, temperature ranged from 25 to 27 °C, salinity was 32‰, dissolved oxygen > 6 mg/L, pH at 8 – 8.4 and NH_3 and NO_2 < 0,02 mg/ L. These parameters are similar to other studies on the culture of barber gobie larvae and other *Elacatinus* species (Olivotto *et al*., 2005; Meireles *et al*., 2009; Shei *et al*., 2010).

Survival

Three and 4 days after hatching it was observed a high mortality in one tank of the non-enriched rotifer treatment. Although not significantly different (P=0.055) survival of larvae reared on n-3 HUFA enriched rotifers was more than three times higher (35.7 ± 3.1%) than larvae reared on non-enriched rotifers (11.1 ± 5.2 %). The high coefficient of variation, 12.1 and 65.9 could have masked the statistical difference on survival rate.

Growth

The standard length (SL) of newly hatched larvae was 3.34 ± 0.07 mm. At 7 dah, larvae fed n-3 HUFA enriched rotifers (4.73 ± 0.09 mm) had grown significantly larger ($P<0.01$) than those reared with non-enriched rotifer (4.5 ± 0.24 mm) (Figure 1). However, at the end of the experiment (14 dah) there was no longer a significant difference of growth for larvae reared on both prey types ($P>0.05$), 6.09 ± 0.62 and 5.69 ± 0.66 mm for larvae fed with n-3 HUFA enriched and non-enriched rotifers respectively.

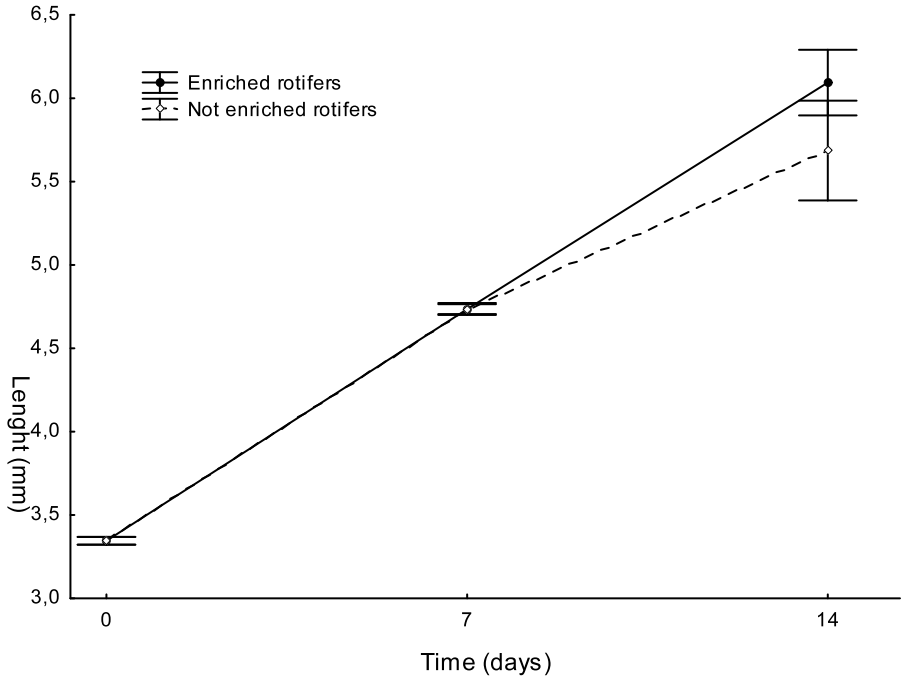

Figure 1: Growth of barber goby larvae *Elacatinus Figaro* fed n-3 HUFA enriched and non-enriched rotifers.

The development of techniques that improve productivity on marine ornamental species will help to produce organisms that commercially compete with less expensive specimens from the wild and thus reduce the pressure on natural stocks (Wabnitiz et al, 2003; Olivotto et al, 2006).

The low fecundity of ornamental species, including barber goby, (Olivotto et al, 2005, Fernando et al, 2006; Meireles et al, 2009) make it difficult to run experiments with the adequate number of replicates. The lack of significant difference of survival and growth at the end of the experiment can be the result of the high coefficient of variation observed for both. Nonetheless, the results strongly suggest that barber goby should be fed *n-3* HUFA enriched rotifer in order to maximize juvenile production.

Although studies have been conducted to determine nutritional requirements and feeding practices to optimize larval production of a number of marine fish species (Sargent *et al*., 1999), studies on marine ornamental species with this type of development are still scarce (Ostrowski and Laidley, 2001; Moorhead and Zeng, 2010).

The essential fatty acids *n-3* HUFA such as DHA and EPA are major components in cell membranes of marine fish and they are also important as source of energy during larval development (Sargent *et al*., 1999; Toucher, 2003). Studies with several species demonstrate better performance in the larviculture with the use of *n-3* HUFA enriched live prey. In fact, growth and survival of seahorses, the false percula *Amphiprion oceallaris* and the Banggai cardinalfish *Pterapogon kauderni* were improved during the early stages of development when fed on n-3 HUFA enriched rotifer and *Artemia* (Chang and Southgat, 2001; Vagelli, 2004). Succesfull production of juvenile sunrise dottyback *Pseudochromis flavivertex* was only achieved with the use of n-3 HUFA enriched live food (Olivotto et al, 2006).

Conclusions The results of this experiment clearly suggest that barber goby larvae should be fed with n-3 HUFA enriched rotifers in order to maximize growth and survival to the juvenile stage.

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Capítulo 03

The development of immune system of the barber goby, *Elacatinus figaro*

Abstract

In teleost fish the head kidney and thymus are generally recognized as important immune organs. This study describes the ontogeny of these organs in barber goby *Elacatinus figaro* larvae and juveniles. Samples were collected periodically between 0 and 94 days after hatch (dah). The description was given by morphometric analysis of the thymus and kidney and phenotypic expression (% / mm^2) of CD3 and CD4 in these organs. At 0 dah it was already possible to observe the kidney and thymus, and they reached respectively 23.1 ± 0.15 mm and 90 ± 1.41 mm at 94 dah. With regard to CD3, the first phenotypic expression was found in thymus at 0 dah and kidney from 6 dah, at 94 dah expressions reached 90 ± 1.41 in the thymus and 53.67 ± 1.53%/ mm^2 in the kidney. The CD4 was initially expressed in the thymus at 3 dah and in kidney at 12 dah reaching respectively 22 ± 1.41 and 44 ± 2%/ mm^2 at 94 dah.

Key words: Neon goby, yellow line goby, fish immune ontogeny, CD3, CD4.

Introduction

The fish immune system in general terms is very similar to that of higher vertebrates (Flajnik, 1996). The immune response of all vertebrates, including fish can be divided into two types: firstly, the unspecific immune response, which is characterized by being antigen independent, as there is no immunological memory. This system acts as a first defense in the presence of a pathogen, and may control various infections without depending on the specific system (Flajnik, 1996; Levraud and Boudino, 2009, Romano 2010, Klosterhoff and Romano, 2012). The second type, the combined response or specific, is inducible and requires the presence of cells that react specifically with the

antigen inducer, the lymphocytes. The designation of a combined response is due to the involvement of two elements: the humoral response mediated by antibody and cellular response, mainly controlled by T lymphocytes (Bernstein et al., 1998; Kourilsky et al., 1998; Levraud and Boudino, 2009).

Kidney and thymus are the major lymphoid organs of teleosts. In freshwater teleosts, the thymus is the first organ to become lymphoid, although earlier the kidney can contain hematopoietic progenitors but not lymphocytes (Lam et al., 2004). In marine teleosts the order in which the major lymphoid organs develop is kidney, spleen, and finally, the thymus, although the larval spleen is more erythropoietic than lymphopoietic (Schroder et al, 1998; Magnadottir et al., 2005; Zapata et al., 2006).

Studies of defense mechanisms are important for successful aquaculture since the diseases are responsible for large economic losses worldwide (Alvarez-Pellitero, 2008). Knowledge of the ontogeny of immune system allows the determination of the moment in which the organism would be able to receive vaccines and also facing more estressful conditions, such as transport handling and the growout period (Kato et al., 2004). Information related to the ontogeny of immune system of fish are restricted and even reduced in marine species (eg. sea bass, Atlantic halibut and Atlantic cod) (Padrós & Crespo, 1996; Zapata et al., 2006).

The gobiidae family is considered the largest among the marine fish, accounting more than 2,000 species and 200 genera (Nelson, 1994). The barber goby, *Elacatinus figaro* is a endemic species of the Brazilian coast, presenting a small size (≥4 cm), easy handling and constant spawning under laboratory conditions. Individuals of this species have an enlogated body, with bright horizontal black and yellow stripes, being of great interest to the ornamental fish market (Sazima et al., 1997; Gasparini et al., 2004; Shei et al., 2010; 2012).

According to this information, this paper presents basic data on the growth of the lymphoid organs and immune development of the barber goby over a period from hatching to 3 months of age.

Materials and Methods

Larvae for this study were obtained from barber gobies broodstock maintened in the Laboratory of Marine Fish Culture of the Universidade federal do Rio Grande – FURG, RS, Brazil (permission SISBIO 22859).

Broodstock pairs were kept in 30 L (40 x 30 x 30 cm) aquariums connected to a recirculating aquaculture system (RAS). All tanks were supplied with a PVC pipe (32 mm diameter and 80 mm length) with an internal plastic film as suitable substrate for egg laying. Fishes were fed four times per day, twice with a commercial dry diet (59 % crude protein, and 16 % lipids, INVE, NRD, Belgium) and twice with frozen *n-3* HUFA enriched *Artemia* metanauplii biomass. Photoperiod was set to 12 h L: 12 h D using fluorescent lighting (6.500 k). Under these conditions, broodstock spawned every 12 days, the clutch size varied between 230 and 340 eggs and the embryo development lasted 7 days.

Larval rearing

Two hours before larvae began to hatch, the plastic film with adhered eggs was removed from the PVC pipe and transferred to a beaker (1 L) with gentle aeration until all larvae hatched. After this, larvae were than split into a 90 L tank (black wall and white bottom) connected to a RAS composed by bag filter (50 μ), two 250 W heaters, a protein skimmer (Plaspiral 800R, Brazil), a fluidized biofilter (bioballs, 5 m^2 total surface) and a 30 W UV sterilizer (> 30.000 $\mu W / cm^2 / s$, Plaspiral, Brazil).

During the larviculture period, the tank was kept under extended photoperiod (24 L: 0 D) and supplied with green water (*Nannochloropsis oculata*, 500,000 cells mL^{-1}).

Through the first 14 days after hatch (dah), larvae were exclusively fed on *n-3* enriched (S Sparkle – Inve, Belgium) rotifers at 10 mL^{-1}, after this (14 – 16 dah) were supplied with *Artemia* nauplii (EG – Inve – USA) between. After 16 dah, until metamorphosis, larvae were fed on *n-3* enriched *Artemia* metanauplii (Easy DC SELCO, Inve - Belgium). After metamorphosis, were offered a dry diet four times a day (INVE, NRD, 400-600 μm, 59% crude protein and 16% lipids).

During the experimental period, broodstock and larval RAS were maintained at: 25 to 27 °C, salinity was 32‰, dissolved oxygen > 6 mg/L (YSI, 55A), pH at 8 – 8.4 (YSI, pH 100 - EUA), NH_3 (UNESCO, 1983) and NO_2 (Bendschneider & Robinson, 1952) <0.02 mg/ L.

Histological analysis.

The histological and immune development were analyzed in the Laboratory of Immunology and Pathology Aquatic Organisms of FURG.

All material was collected from a single clutch. Samples of three larvae and juveniles were collected after hatching and at 1, 2, 3, 6, 9, 12, 15, 18, 20, 23, 26, 30, 40, 49 and 94 dah. All fish were anesthetized with benzocaine (30 ppm) before fixed in bouin's solution. After this the samples were embedded in paraffin wax, sectioned in 4 μm and stained with haematoxylin – eosin and PAS. Slides were observed and photographed (Olympus, DP 72 digital camera) under a microscope (Olympus, BX45).

Was evaluated the development of lymphoid organs (thymus and kidney), the assessment was established by the presence or absence of these organs. When present, a morphometric analysis was performed.

Immunohistochemistry

The immunohistochemistry of the lymphoid organs were performed using the same samples processed for the histological analyzes. Immunohistochemistry was performed by avidin-biotin method modified (Vectastain Elite, Vector). The sections were incubated for 90 minutes with a monoclonal anti-CD3 and anti-CD4 (DAKO, Argentina) at a dilution of 1:1000 in PBS, then washed in PBS and exposed for 45 minutes to avidin biotin - peroxidase (HSUS, 1981). Thereafter the sections were washed (0.1% solution diaminobenzidine, Sigma), dried and the slides were mounted and examined under an optical microscope. As for the evaluation of CD3 and CD4 receptors were carried out the quantitative analysis of phenotypic percentage of tissue per square millimeter (Weibel, 1962; Romano et al 1996).

Data Analysis.

The length and area of the lymphoid organs were charted using Statistica 7.0 Software®. Results are expressed in mean ± standard deviation of the data.

Results

Growth

The larvae and juveniles of *E. figaro* showed a linear regression growth during the study period. The newly hatched larvae measured 3.51 ± 0.15 mm and began to settle to the bottom of the tanks from 33 dah, when the organisms measured about 8.5 ± 0.06 mm. At 94 dah, juveniles possessed 18.41 ± 0.17 mm (Figure 1).

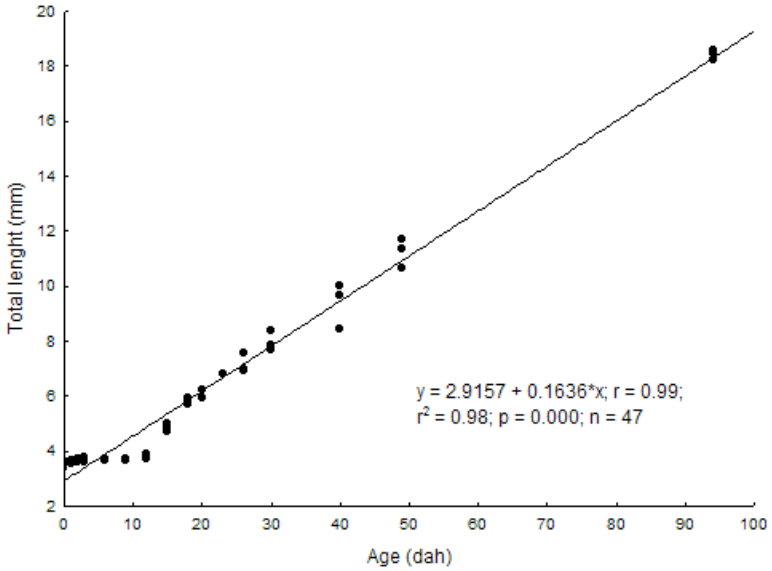

Figure 1: Barber goby growth between 0 to 94 days after hatching.

At time of hatching, the head kidney was already present, measuring 0.01 mm ± 0.31 (Table 1) and presenting tubular structures and groups of lympho hematopoietic cells. The tissue mass followed hematopoietic growth of animals and at 6 dah (3.69 ± 0.03 mm), showed the greatest proliferation of tubules with isolated glomerulus. In the head kidney it was observed abundant lympho hematopoietic cells. At 30 dah (7.96 ± 0.36 mm), the kidney is fully developed, clearly differentiating the posterior kidney and the head kidney, the last one with less tubules and a significant increase in lympho hematopoietic cells between tubules. At 94 dah (18.41 ± 0.17 mm), the kidney appears completely developed, glomerulus and tubules with little hematopoietic activity in the posterior kidney. In contrast, few tubules are observed in head kidney, with isolated glomerulus and lympho hematopoietic cells with capillary sinusoids.

The thymus was observed at 0 DAH, and at this moment it measured 0.03 ± 0.03 mm. There is the beginning of continuous tissue that covers the gill chamber and demonstrates few lymphocytes surrounded by a slender fibrous capsule. At 6 DAH (3.69 ± 0.03 mm) the thymus already is a structure with well-established lymphoid cells

occupying the wall of branchial chamber. On the 30th DAH (7.96 ± 0.36 mm), it is observed an increase in lymphoid mass, growing in the periphery of branchial chambers. At 94 DAH (18.41 ± 0.17 mm), the thymus measured 0.09 ± 0 mm (table 1), being fully developed, with the fibrous capsule attached to the epithelium of the gill chamber.

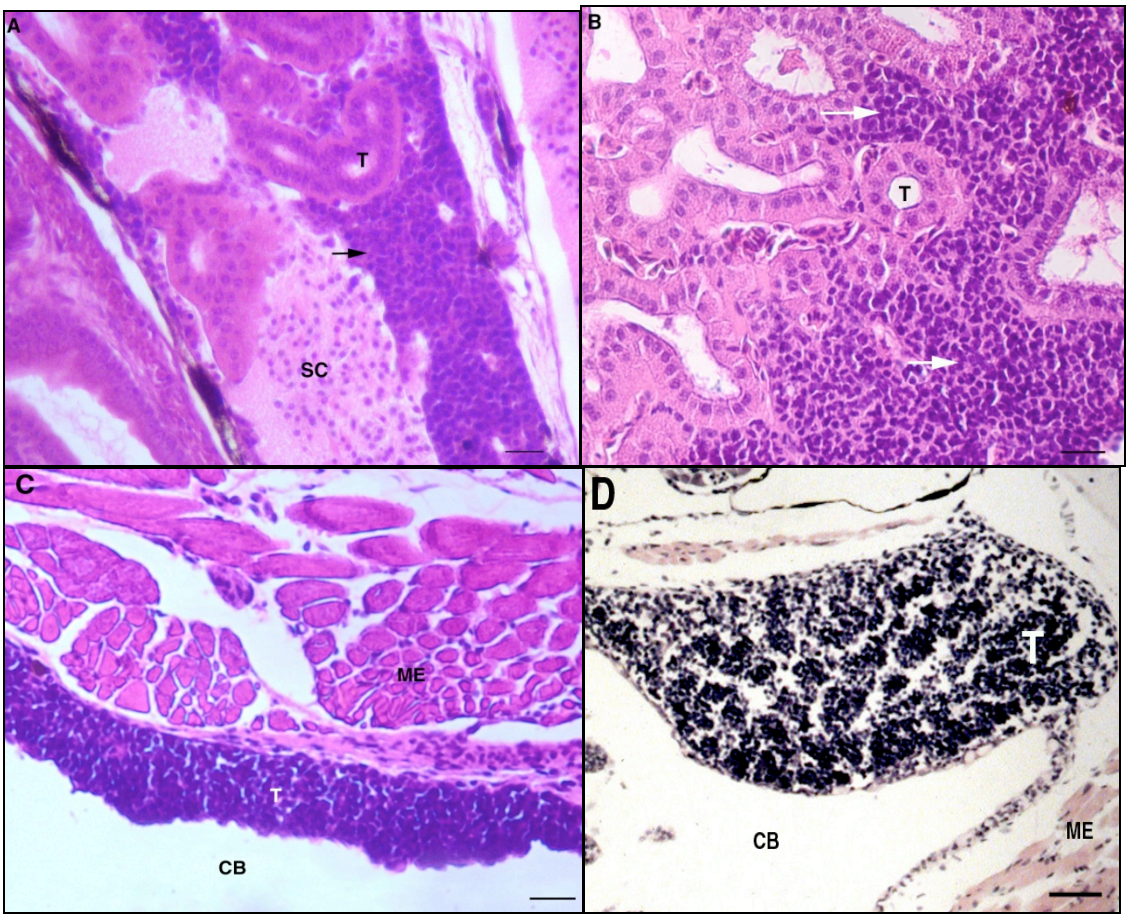

Figure 2: A: Larval kidney at 6 dah with tubules (T), capillary sinusoids with erythrocytes (SC) and lymph hematopoietic cells (arrow) HE (bar = 20 micrometers. O 20 X). B: Kidney of 94 dah Juvenile with tubules (T), and abundant lympho hematopoietic cells (arrow) HE (bar = 20 micrometers. O 20 X). C: Larvae at 6 dah, which is observed thymus (T) projecting on the gill chamber (CB) and skeletal muscle (ME). H-E (Bar = 10 mm. O 10 X). D: Juvenile at 94 dah, where we observe the thymus (T)) reaching the gill chamber (CB) and underlying skeletal muscle (ME). H-E (Bar = 10 mm. O 10 X)

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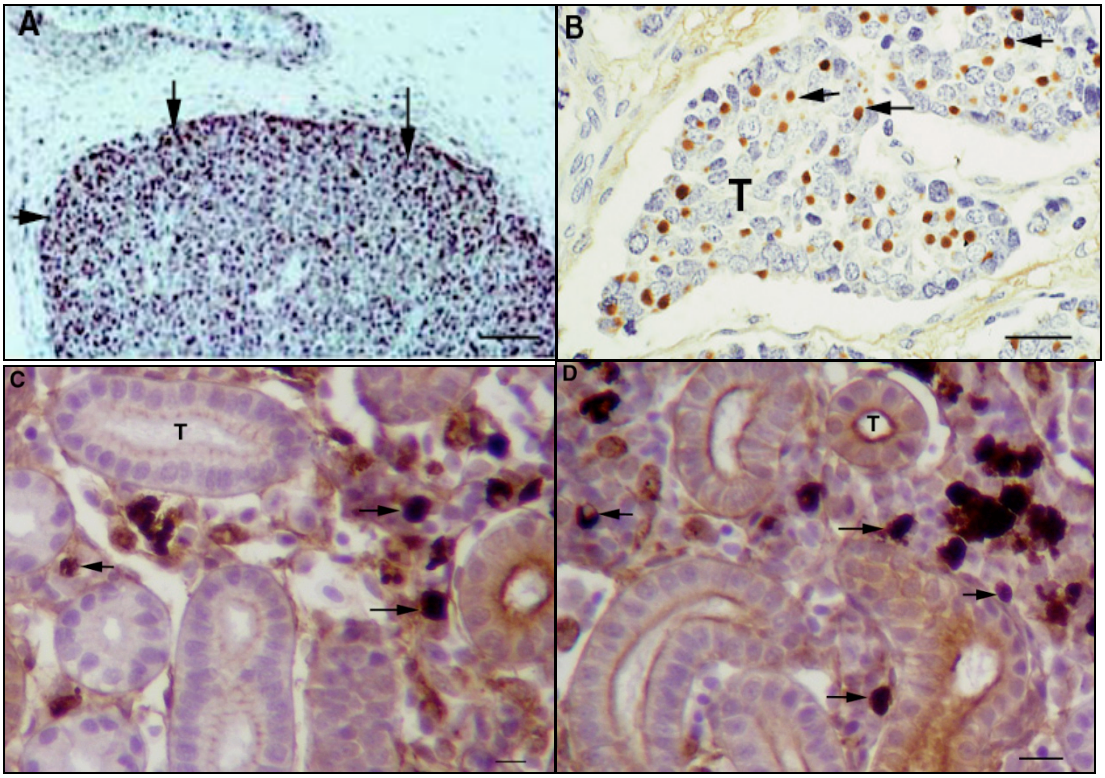

Figure 3: A: Thymus with abundant lymphocytes stained with anti CD3 (arrows). Anti-CD3 (bar = 20 micrometers. O 20 X). B: Thymus (T) lymphocytes stained with anti-CD4 (arrows) (Bar = 10 mm. O 20 X). C: Kidney observed in the lymph hematopoietic tissue surrounding the tubular (T) lymphocytes stained with anti-CD3 (arrows). Anti CD3 (bar = 50 micrometers. O 40 X). D: where kidney observed in the lymph hematopoietic tissue surrounding the tubular (T) lymphocytes stained with anti-CD4 (arrow). Anti CD4 (bar = 50 micrometers. O 40 X).

CD3 Expression

The first appearance of phenotypic expression of lymphocyte CD3 receptor were recorded in the thymus tissue of newly hatched larvae (0 dah), expressing 71.25 ± 8.82% / mm ^{2}, when the animals measured 3.51 ± 0.15 mm (Figure 4). In the head kidney tissue, the first record of CD3 expression occurred at 6 dah, when the larvae measured 3.69 ± 0.03 mm, starting at 2 ± 0.0 % / mm ^{2} (Figure 4). During the study period there was a continuos increase in lymphocytes immunostained with anti-CD3 and the highest concentration occurred at 94 dah, when juvenile measured 18.41 ± 0.17 mm in length, expressing 90.00 ± 1.41 and 53.67 ± 1.53% / mm^2 in the thymus and head kidney respectively (Table 1).

CD4 Expression

The expression of CD4 (T helper) was observed firstly in thymus, representing 1.33 ± 1.15% / mm^2 at 3 dah when larvae measured 3.68 ± 0.07 mm (Figure 5). In head kidney, the first occurrence was found at 12 dah when larvae measured 3.83 ± 0.10 mm, demonstrating 5.33 ± 1.53% / mm^2 (figure 5). During the period analyzed, the phenotypic expression of CD4 in thymus and head kidney tissue continued to increase, reaching respectively 22 ± 1.41 and 44 ± 2.0% / mm^2 at 94 dah, when the juveniles measured 18.41 ± 0.17 mm (Table 1).

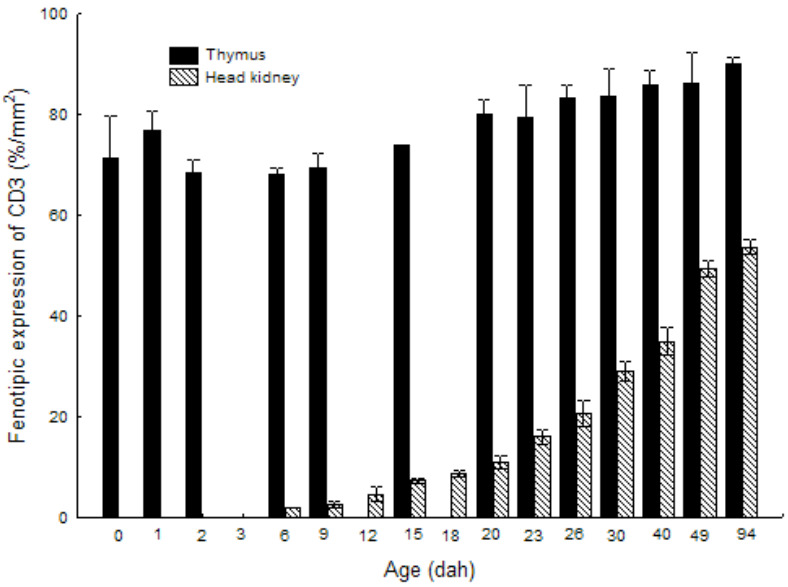

Figure 4: Fenotipic expresion of CD3 marker in thymus and head kidney of barber goby during the study period.

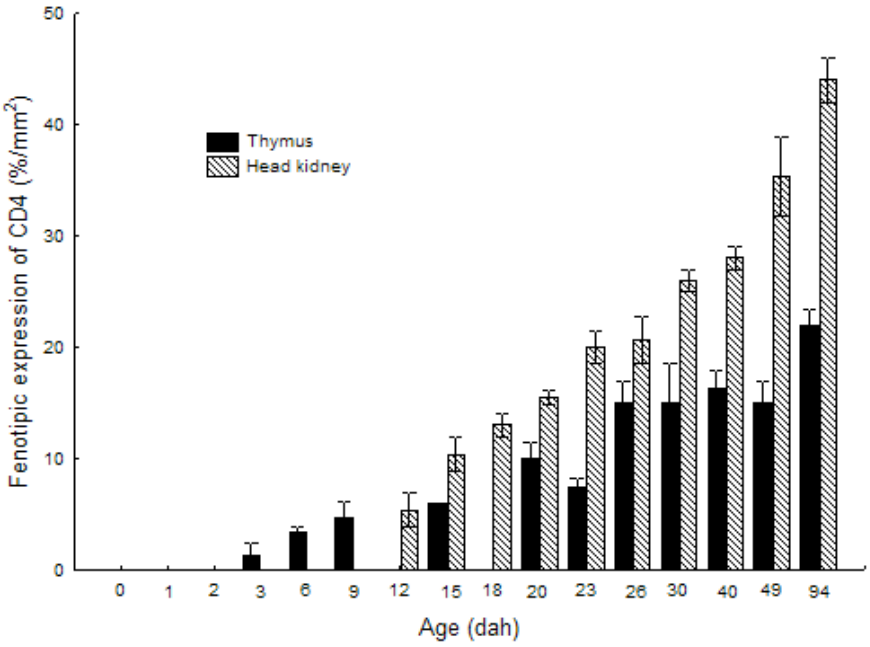

Figure 5: Fenotipic expresion of CD4 marker in thymus and head kidney of barber goby during the study period.

Table 1: Age, Lenght, CD3 and CD4 fenotipic expression (μm^2) on tymus and head kidney in barber goby. Mean ± SD.

Age (days after hatching)	Total lenght (mm)	Thymus lenght (mm)	% CD3 Thymus	% CD4 Thymus	Head kidney length (mm).	% CD3 Head kidney.	% CD4 Head kidney.
0	3.51 ± 0.15	0.03± 0.03	71.25 ± 8.42	0	0.31 ± 0.01	0	0
1	3.65 ± 0.07	0.03 ± 0.02	76.67 ± 4.04	0	0.36 ± 0.02	0	0
2	3.64 ± 0.07	0.03 ± 0.01	68.33 ±2.52	0	0.35 ± 0.05	0	0
3	3.68 ± 0.07	NA	NA	1.33 ± 1.15	0.34 ± 0.02	0	0
6	3.69 ± 0.03	0.05 ± 0.01	68 ± 1.41	3.33 ± 0.58	0.33 ± 0.01	2	0
9	3.7 ± 0.01	0.06 ± 0	69.33 ± 2.89	4.67 ± 1.53	0.33 ± 0.02	2.67 ± 0.58	0
12	3.83 ± 0.10	NA	NA	NA	0.37 ± 0.05	4.67 ± 1.53	5.33 ± 1.53
15	4.86 ± 0.13	0.06	74	6	0.32 ± 0.01	7.33 ± 0,58	10.33 ± 1.53
18	5.83 ± 0.11	NA	NA	NA	0.48 ± 0.06	8.67 ± 0.58	13 ± 1
20	6.08 ± 0.22	0.03 ± 0.02	80 ± 2.83	10 ± 1.41	0.53 ± 0.01	11 ± 1.41	15.5 ± 0.71
23	6.81 ± 0.04	0.04 ± 0.01	79.5 ± 6.36	7.5 ± 0.71	0.55 ± 0.04	16 ± 1.41	20 ± 1.41
26	7.15 ± 0.04	0.04 ± 0.03	83.33 ± 2.52	15 ± 2	0.6 ± 0.03	20.67 ± 2.52	20.67 ± 2.08
30	7.96 ± 0.36	0.07 ± 0.01	83.67 ± 5.51	15 ± 3.61	0.68 ± 0.03	29 ± 2	26 ± 1
40	9.36 ± 0.82	0.06 ± 0.01	85.67 ± 3.06	16.33 ± 1.53	0.82 ± 0.02	35 ± 2.65	28 ± 1
49	11.22 ± 0.54	0.07 ± 0.01	86 ± 6.24	15 ± 2	0.91 ± 0.03	49.33 ± 1.53	35.33 ± 3.51
94	18.41 ± 0.17	0.09 ± 0	90 ± 1.41	22 ± 1.41	1.23 ± 0.15	53.67 ± 1.53	44 ± 2

Discusssion

The growth rate of barber goby larvae in the present study was similar to those described by Meirelles et al. (2009) and Shei et al. (2010). However, this is the first study to present data of captive bred barber goby growth after metamorphosis.

The immune system of teleost fish shows several characteristics commun to mammals, especially as the cellular and molecular structure. Differences are given by the absence of lymphatic glands and bone marrow, the latter being developed on fish head kidney, having hematopoietic and part of lymphoid function (Zapata et al., 1996; 2006). In recent years, histological descriptions of the ontogenetic development of the lymphoid organs have been made for teleost fishes although differences occur between species (Romano et al., 1999; Kato et al., 2004; Alvarez-Pellitero, 2008).

Upon hatching, the kidney presented mainly excretion tubules, and 6 dah, have presented abundant amounts of lympho hematopoietic cells and the expression of CD3. At 12 dah (3.83 ± 0.10 mm), it was determined the moment that occurs the reestablishment of T lymphocytes in head kidney, established through the positive expression of CD4 and is characterized as a lymphoid organ. This process has already been reported in the earlier stages of development of European eel *Anguilla anguilla* (Nielsen & Esteve-Gassent, 2006), the Asian sea bass, *Lates cacarifer* (Azad et al, 2009) and the Atlantic halibut, *Hipoglossus hipoglossus* (Patel et al., 2009).

Thymus is thought to be the major organ for storage and maturation of T cells and the first lymphoid organ that acquires lymphocytes during the histogenesis of the lymphoid tissue (Manning, 1994; Zapata et al, 1996). In the present study, the thymus was present at 0 dah and became lymphoid at 3 dah (3.68 ± 0.07 mm), being recognized by the expression of CD4 marker. Despite the thymus has a lower initial development, as already described for other marine species, the thymus started to have

an accelerated development and became lymphoid prior the head kidney (Padrós and Crespo, 1996, Schroder et al., 1998, Liu et al., 2004; Patel et al., 2009). Unlike the reproductive strategy of most marine fish species, the barber goby has a low fecundity with large eggs and a long embryonic development (Shei et al., 2010). This may explain the fact that besides the kidney, the thymus was also already present at the time of hatching.

The small number of studied species and the differences of the lymphoid organs in marine and fresh water species highlights the need for a marine species that can be used as experimental model, such as zebrafish *Danio rerio* (Grunwald and Eisen, 2002; Magnadottir et al., 2005; Zapata et al., 2006). The barber goby is a species with great interest in the international marine ornamental market (Gasparini et al., 2004) and studies like this collaborate for their production and also demonstrate the possible use of this species as a model for several studies with marine fish.

The barber goby has a rapid development of lymphoid organs, with morphologic and functionally maturation in the first feeding period. Despite an expected positive correlation between the formation of lymphoid organs, the expression of immune markers and immunecompetence (Patel et al., 2009), specific studies should be conducted to determine the time at which organisms would be able to receive vaccines and cope with stressful situations as intensive growout and transport handling.

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DISCUSSÃO GERAL

Os recifes de corais abrangem menos de 1 % do ambiente marinho e são considerados os ecossistemas com maior biodiversidade, produtividade e complexidade no planeta (Birkeland 1997; Paulay, 1997). Apesar de não haver qualquer estudo caracterizando o status do barber goby como em risco de extinção, motivo esse usado para alegar a proteção e proibição de coleta da espécie, o presente trabalho colabora para o desenvolvimento de um comércio mais responsável, conciliando o aumento do mercado sem a necessidade de atividades extrativistas.

Quanto a performance reprodutiva, estudos relacionam a deficência nutricional de *n-3* HUFA com dos índices reprodutivos inferiores, expressa na qualidade de ovos e larvas, enquanto a resposta na fecundidade varia de acordo com as espécies (Furuita et al., 2002; 2007; Callan et al., 2011). Assim como os efeitos relacionados a deficiência de ácidos graxos essenciais, o seu excesso também tem sido relacionado com efeitos negativos na performance reprodutiva de peixes (Izquierdo et al., 2001, Furuita et al., 2007).

No presente estudo, independentemente da suplementação com *n-3* HUFA, as dietas foram capazes de condicionar os reprodutores a desovar continuamente durante os quatro meses, não sendo constatadas diferenças significativas na frequência de desovas e volume dos ovos. A suplementação com *n-3* HUFA resultou em valores significativamente menores, também demonstrados por Fernandez-Palacios et al. (1995) em estudos com o pargo europeu, onde peixes alimentados com uma dieta contendo 3,5 % de *n-3* HUFA demonstrou uma diminuição no número de ovos liberados. Com isso, pode-se presumir que o total de lipídeos (16 %) e o perfil contido na ração comercial utilizada e na biomassa de *Artemia* são suficientes para condicionar o barber goby a desovar, sendo que e a suplementação ofertada através da biomassa de

Artemia enriquecida com *n-3* HUFA excedeu o nível ótimo para a espécie. Com isso, sugere-se que estudos dosando diferentes quantidades de *n-3* HUFA na dieta sejam realizados de modo a otimizar o manejo reprodutivo dessa espécie.

Quanto a sua criação nas idades iniciais, a utilização de enriquecimento com *n-3* HUFA no alimento-vivo tem resultado em melhores resultados durante a larvicultura de várias espécies. Foi descrito índices de crescimento e sobrevivência superiores para o peixe palhaço, *Amphiprion oceallaris* (Chang e Southgat, 2001) e o Banggai cardinalfish, *Pterapogon kauderni* (Vagelli, 2004), enquanto que o sucesso na produção de juvenis do sunrise dottyback, *Pseudochromis flavivertex* só foi alcançado com a utilização de enriquecimento do alimento vivo (Olivotto et al., 2006).

No presente trabalho, a utilização de rotíferos enriquecidos com *n-3* HUFA correspondeu a uma taxa de sobrevivência mais elevada (35,7 %) do que o tratamento que não recebeu rotíferos enriquecidos (11,1 %). Resultados similares na taxa de sobrevivência (32 %) também foram encontrados por Côrtes e Tsuzuki (2011) em testes experimentais que utilizaram de rotíferos enriquecidos e outros alimentos-vivos na primeira alimentação do barber goby.

A baixa fecundidade de espécies de peixes ornamentais marinhas, incluindo o barber goby (Olivotto et al, 2005, Fernando et al, 2006; Meireles et al, 2009) torna difícil a realização de experimentos com o número adequado de réplicas. A ausência de diferenças significativas nas taxas de sobreviência e crescimento ao final do experimento provavelmente seja ao altos coeficientes de variação encontrado em ambos tratamentos. Independentemente, os resultados sugerem que as larvas do barber goby devem ser alimentados com rotíferos enriquecidos de modo a maximizar a produção de juvenis.

O sistema imune de peixes teleósteos possuem características comuns aos mamíferos, especialmente a estrutura celular e molecular. As diferenças são devidas a ausência de gânglios linfáticos e da medula óssea, sendo este último, homólogo ao rim anterior dos peixes, realizando funções hematopoiéticas e linfóides (Zapata et al, 1996;. 2006). Nos últimos anos, descrições do desenvolvimento ontogenético dos órgãos linfóides têm sido feitas para peixes teleósteos, embora as diferenças podem ocorrer entre espécies (Romano et al, 1999;. Kato et al, 2004;. Alvarez-Pellitero, 2008).

O barber goby possui um acelerado desenvolvimento dos órgãos linfóides, com a funcionabilidade morfológica e maturação durante o período da primeira alimentação. Apesar de se esperar correlações positivas entre a formação dos órgãos linfóides, a expressão dos imunomarcadores e a imunocompetência, estudos específicos devem ser realizados para determinar o momento em que os organismos poderiam receber vacinas e se deparar com situações mais estressantes quando mantidos em sistemas intensivos de crescimento e manejo de transporte.

A facilidade de se obter desovas constínuas por um período prolongado com o uso de dietas comerciais e a viabilidade de produção de juvenis em uma estrutura compacta, candidata o barber goby como uma espécie marinha modelo experimental, como ocorre com o zebrafish *Danio rerio* (Grunwald & Eisen, 2002).

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