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Original Article

Effect of protein and lipids levels in a growth diet on adult whitebait *Galaxias maculatus* (Jenyns 1842)

Efeito dos níveis de proteína e lipídio em uma dieta de crescimento em whitebait Galaxias maculatus adulto (Jenyns 1842)

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Abstract

Galaxias maculatus aquaculture objectives is to produce millions of eggs. Wild females are small (2 g), have quick sexual maturity and low mean fecundity (500 eggs/female), requiring larger fishes with higher fecundity. This study aim is to evaluate experimentally the effect of the levels of protein, lipid and dietary energy on weight increases in adults. Five independent experiments were performed at different sequential time periods at the UCT hatchery, Chile. Specimens were obtained from a) Crystalline sea return specimen catches in the Tolten estuary (4 -6 cm, 0.3-0.4 g.). b) Hatchery cultured fish. Fish were fed by hand *ad libitum*. In experiments 1 to 4, pelleted diets were prepared with 3 to 5 levels of protein (treatments 27 up to 57%), crumble size, three 100 L fibre ponds replicates. In experiment 5 the effect of two lipid levels (8 and 21%) was evaluated with commercial extruded Salmon Nutra Starter isoproteic crumble 1 diet at 63%, replicated in 4 ponds. The results show: A tendency to increased weight in all sizes with an increased protein level in the pelleted diet.A maximal adult growth is obtained with a diet containing a minimum of 37% crude protein, with 40% the optimal value. A higher % protein in the diet or growth in weight lower feed conversion ratio. The feed conversion ratio in the extruded diet reaches up to 0.5 and in the pelleted vary from 0.7 to 1.5. Fish 0.6 g fed with 63% protein, extruded commercial diet with two different lipid levels (8 and 21%, 20.40 and 23.84 MJ kg^{-1}, PE/TE 0.62 and 0.71) increased weight the first month 67 and 105% each. It has been established that high-energy diets with optimal levels of protein and lipid are a good short-term solution to obtain *G. maculatus* of higher weight.

Keywords: *Galaxias maculatus*, protein diet, lipid diet, growth.

Resumo

O objetivo da aquicultura de *Galaxias maculatus* é produzir milhões de ovos. As fêmeas selvagens são pequenas (2 g) e têm maturidade sexual rápida e fecundidade média baixa (500 ovos/fêmea), necessitando de peixes maiores e com fecundidade superior. O objetivo deste estudo é avaliar experimentalmente o efeito dos níveis de proteínas, lipídios e energia da dieta sobre o aumento de peso em adultos. Cinco experimentos independentes foram realizados em diferentes períodos sequenciais de tempo no incubatório UCT, Chile. Os espécimes foram obtidos a partir de: a) capturas de espécimes de retorno do mar cristalino no estuário de Tolten (4-6 cm, 0,3-0,4 g); b) peixes de cultura em incubatório. Os peixes foram alimentados à mão ad libitum. Nos experimentos de 1 a 4, dietas peletizadas foram preparadas com três a cinco níveis de proteína (tratamentos 27 a 57%), tamanho do crumble, três repetições de tanques de fibra de 100 L. No experimento 5, o efeito de dois níveis de lipídios (8 e 21%) foi avaliado com dieta comercial isoproteica crumble 1 de Salmon Nutra Starter extrusada a 63%, replicada em quatro tanques. Os resultados mostram: uma tendência ao aumento de peso em todos os tamanhos, com um aumento do nível de proteína na dieta peletizada; um crescimento adulto máximo com uma dieta contendo um mínimo de 37% de proteína bruta, com 40% do valor ideal; uma porcentagem maior de proteína na dieta ou crescimento em peso com menor taxa de conversão alimentar. A taxa de conversão alimentar na dieta extrusada chega a 0,5, e na peletizada varia de 0,7 a 1,5. Peixes de 0,6 g alimentados com 63% de proteína e dieta comercial extrusada com dois níveis lipídicos diferentes (8 e 21%; 20,40 e 23,84 MJ kg-1; PE / TE 0,62 e 0,71) aumentaram de peso no primeiro mês em 67 e 105% cada, respectivamente. Foi estabelecido que dietas de alta energia com níveis ótimos de proteínas e lipídios são uma boa solução de curto prazo para obter G. maculatus de peso mais alto.

Palavras-chave: *Galaxias maculatus*, dieta proteica, dieta lipídica, crescimento.

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1. Introduction

One of the primary challenges and targets of *Galaxias maculatus* (Jenyns, 1842) culture or "galaxy culture" is to produce millions of eggs, since 3,000 fingerlings per kilogram of product are required. Therefore, during their six month immature phase, maximum weight and maturity must be reached, so that the fish will mature and spawn periodically. Wild females are small (1 to 5 g), quickly mature sexually over one year and of low fecundity (500 eggs/female). In experimental cultures, heavier specimens with higher fecundity were obtained (5,000 to 10,000 eggs/female) when required (Mitchell, 1989; Barile et al., 2003, Mardones et al., 2008). Barile et al. (2003) notes that with increasing female weight, the number of eggs increases according to the equation: Number eggs = 305.5 $W^{1,0712}$. Therefore, the challenge is to obtain heavier females. The formulation of energy-rich diets with optimal levels of proteins and lipids constitutes a solution to obtain higher weights and fecundity in *G. maculatus* females in the short term.

G. maculatus is a carnivorous fish. The larvae feed on zooplankton and the adults on arthropods and other benthic organisms. To cultivate breeders in experimental stages, semi-intensive systems are used, based on natural feeding complemented with salmon-extruded food, which generates a quick growth in weight and size, resulting in a higher female fecundity and therefore an increased production of eggs (Vega et al., 2013).

Nutrition has an appreciable effect on gonad activity, increasing growth and fish fecundity (Bromage, 1995) and affecting several reproductive aspects such as time to sexual maturity; fertility, size and quality of the egg, assessed by their chemical composition; and larval survival (De Silva and Anderson, 1995). Therefore, special attention has been devoted to the dietary components, with protein one of the most important because it is one of the most abundant in the eggs of many species (Watanabe et al., 1985).

One of the most important energy sources for fish is proteins, especially in carnivorous fishes, who have a strong dependence on a high protein diet component, because of their ability to digest and metabolize protein compared to carbohydrates (Cowey and Sargent, 1979; Tacon and Cowey, 1985; Cho and Kaushik, 1990; Cho, 1992; NRC, 2011). In high quality fishmeal, protein is the main ingredient in the cultured carnivorous fish diet, as it affects consumption, digestibility and growth (Aksnes, 1995; Aksnes et al., 1997; Vergara et al., 1999).

The optimization of dietary protein use is associated with an improved feed efficiency (Shapawi et al., 2014; Jiang et al., 2016; Wang et al., 2016). Because proteins are increasingly expensive, lipid levels have increased in fish feed production, used as an energy source to save protein and improve the feed conversion ratios. The main effect of these savings in dietary lipids (sparing effect) is to replace a fraction of the protein that otherwise would be catabolized and used as an energy source or to synthesize lipids (Vergara et al., 1996a; Company et al., 1999). Other approaches optimize the use of protein minimum levels in the diet, either for maximum growth in various species of farmed fish (Vergara et al., 1996b; Pérez et al., 1997; Schuchardt et al., 2008) or as energy in respect to the total diet energy (Santinha et al., 1999; Skalli et al., 2004; Jiang et al., 2016).

The increase in lipids to between 20 to 30% in fish diets has led to an increase in gross energy up to 22 to 25 MJ kg^{-1} (Vergara et al., 1999; Peres and Oliva Teles, 1999; Boujard et al., 2004; García-Meilan et al., 2016). This is possible only by the diet extrusion technique, a process that improves the digestibility of proteins, lipids and carbohydrates, promoting a better growth than pelleted diets (Deguara, 1997; Pérez et al., 1997). Carbohydrates, due to their relative digestibility in carnivorous fishes, bind and increase the palatability and flavour of the food (Jeong et al., 1991).

Artificial feeding studies in *G. maculatus* future breeders are just beginning and are essential to establish the optimal dietary levels of protein and lipid for maximum growth in weight and fecundity. The aim of this study is to evaluate the effect of protein, lipids and energy in pelleted and extruded diets on adult growth in *G. maculatus.*

2. Materials and Methods

2.1. Diets and experimental design

To evaluate the effect of protein, lipid and energy in the diets on diadromic adult weight in *G. maculatus* (Jenyns, 1842), five independent experiments were performed at different sequential periods of time at the School of Aquaculture, Universidad Catolica de Temuco hatchery, Araucania Region, Chile. Four evaluated the effect of protein and energy levels in the diet and the fifth evaluated the effect of lipid and energy levels in the diet. The characteristics of the fish (numbers of ponds and fish, origin: wild or farmed, start weight range and month of experiment duration) are shown in Table 1. The experiments were repeated, except for experiment 1, due to fish maturation and mortality. The experiments are labelled as 2.1 (the first) and 2.2 (its repeat), for example.

Specimens were obtained from a) Crystalline sea return specimen catches in the Tolten estuary of Chile´s Araucania Region between September and November, from 4 to 6 cm of total length and 0.3 to 0.4 g. The fish were adapted to the diet over one month by gradually replacing bovine liver feed with formulated pelleted feed. b) Hatchery cultured fish in a 100 L fibre pond with well-aerated water with an exchange rate of 0.5. The fish were feed by hand *ad libitum* three times a day six days a week, avoiding wasting food in the pond bottom.

In experiments (Exp.) 1 to 4, diets prepared with 3 to 5 levels of protein (treatments) and crumble size N° 1 (850 microns), the effect of different protein levels was evaluated with isocaloric (similar caloric value) pelleted diet with at least 27% up to 57%. Each treatment had three ponds replicates. On this diet, the energy remained constant, and the protein and lipid per cents were balanced, increasing protein (fishmeal) and reducing lipid (fish oil). The diet ingredients such as "candaline binder" (durum wheat groats), meal and high quality fish oil used in salmon food were supplied by Skretting, Puerto Montt, Chile, and the vitamin supplements and minerals were supplied by Veterquimica, Santiago, Chile. Vita size 1 from Salmofood SA (Ext.V1) salmon commercial extruded diets were used as the reference diet. Per cent protein and lipid of the experiment diets:

Exps. 1 and 2.1: Pelleted Diets 1 to 3 with 27, 32, 37% protein;

Exps. 2.2, 3 and 4: Pelleted Diets 1 to 5. Pelleted diet 4 has a higher protein level (56-57%) and extruded Diet 5 was in:

a) Exp. 2.2: 53% protein (NS: Salmon commercial Nutra Starter Trouw Chile, Skretting);
b) Exps. 3.1 and 3.2: 58% protein (Ext. V1);
c) Exps. 4.1 and 4.2: 55% protein (Ext. V1).

The effect of different lipid levels was evaluated with extruded Salmon Nutra Starter (NS) isoproteic crumble 1 diet at 63% of experiment 5, replicated in 4 ponds. The NS commercial diet is 21% lipid, which was compared with 8% by not adding fish oil to the diet.

2.2. Pelletized diet elaboration methodology

Fishmeal and "candealine binder" were sieved to 2000, 850 and 600 microns. The estimated quantities of each ingredient were weighed and homogenized (fishmeal, vitamin and minerals premix, vitamin C) in a metallic container with a KitchenAid K5SS, USA, mixer for 30 min. Then, the liquid ingredients were added; that is, fish oil and a mix of "candealine binder" with distilled water (900 mL water kg^{-1}). This mixture was boiled and stirred over low heat for 15 min. and then passed through a 1 HP Super Chacon (Chile) laboratory mixer, with a two or five mm gauge opening. The mixture was cold pressed for one hour until a homogeneous paste was obtained, pelletized and placed on trays covered with aluminium foil. The pellets were heated in a Memmert oven (Germany) at 60°C for 48 hours before being fragmented by a Moulinex grinder (France) and sifted to obtain the desired size of pellet granules (crumble 0: 600 microns, Vita 1 or crumble 1: 850 microns). The compositions of the experimental diets are shown in Table 2.

2.3. Proximate analysis of pelletized diets

The percentages of dry matter were determined by a gravimetric method after drying the pellets in a 105°C oven to constant weight. The total ash percentage was estimated by incinerating the samples in a Ney Vulcan A550, muffle (USA) at 600°C for 6 hours. The percentages of crude protein were determined (N × 6.25) using the Kjeldahl method (AOAC, 1995) and the total lipid percentage was determined using a petroleum ether extraction method and the Soxhlet technique (Folch et al., 1957). Crude fibre was estimated by a double acid/alkaline attack (Windham, 1995). Carbohydrates and gross energy were estimated using Equations 1 and 2 (Maynard and Loosli, 1979):

Table 1. Number of ponds, experiment duration, number of fish, status, source and start weight range of *G. maculatus* specimens used in the five feeding experiment.

Exp. N°	N° pond	Duration (month)	N° fish/ pond	Status and Source	Start weight range (g)
1.0	18	5	100	Wild crystalline	0.4
2.1	18	5	50	Wild adult	1.4
2.2	15	3	100	Wild adult	3.9
3.1	15	3	150	Wild adult	1.1-1.8
3.2	15	3	200	Wild adult	1.4-2.0
4.1	15	3	100	Wild crystalline	0.5
4.2	15	3	100	Wild adult	1.3-1.6
5.0	8	3	225	Farmed crystalline	0.6

Table 2. Percentages of ingredients for the isocaloric pelleted diets with different percentages of fishmeal used in *G. maculatus* adult feeding experiments.

Ingredients (%)	Diet			
	1	2	3	4
Fishmeal	20	35	51	67
Fish oil	22	22	15	9
Candealine	57	43	33	23
Vitamin/minerals Premix	0.5	0.5	0.5	0.5
Vitamin C (ascorbic acid)	0.5	0.5	0.5	0.5
Total	100	100	100	100

$$Carbohydrates\ (ENN) = 100\% - protein - lipid - ashes - fibre \quad (1)$$

$$Gross\ Energy = 23.6\ MJ\ kg^{-1} \times \%\ protein + 39.8\ MJ\ kg^{-1} \times \%\ lipid + 17.2\ MJ\ kg^{-1} \times \%\ carbohydrates / 100 \quad (2)$$

The results of the proximate analyses of the diets are shown in Table 3.

2.4. Registration and measurement of parameters

Minimum and maximum water pond temperature (ºC) and fish mortality were registered daily and used to calculate the daily mean temperature, % of monthly mortality and % of accumulated mortality. Fish were anesthetized with 0.3 ml benzocaine BZ 20 L^{-1} and monthly sampled by estimating the weight of 25 specimens per pond, which represents more than 5% of the fish, using an Ohaus (USA) balance with 0.1 g accuracy. Fish growth was estimated by using the following parameters: Monthly increase percentage in weight; daily increased percentage in weight; and Growth Factor 3. To evaluate monthly food efficiency, the Feed Conversion Ratio was used following the Dantagnan et al. (2012) methodology. Parameter averages, standard deviations and the coefficient of variation percentage from 3 replicates were also calculated. The crude protein energy ratio/total energy of the diet was estimated (Lee and Putnam, 1973) and the sexual maturity stages of *G. maculatus* specimens were determined using the macroscopic scale method of Valdebenito and Vega (2003).

2.5. Statistical analysis

From the results of % monthly weight growth, only the first month from each treatment in experiments 1 to 4 were statistically analysed. The variability in the results from the second month is explained in the discussion. In experiment 5, the first and second month were analysed. The results of % monthly weight growth (Table 4), previously an arcsine transformation, were compared by one way analysis of variance (ANOVA) followed by multiple comparisons between means using Tukey's test to detect differences (Zar, 1984). To estimate the equations in Table 5 of the logarithmic relationships in protein percentage and protein energy/total energy diets and monthly weight growth rate for different weights of *G. maculatus* the data used come from Table 4. The protein optimal percentage in the diet was estimated by calculating the point where the straight 1 (%Growth = 7.6481 + 0.3153% Protein) and 2 (%Growth = - 1.8592 + 0.5542% Protein) are intercepted, and estimated by regression of the coordinates of the lower half and upper curve (%Growth = - 42.344 + 16.81% Protein).

3. Results

3.1. Effect of different levels of protein and energy on growth to adult weight in Galaxias maculatus

The results show a tendency to increased weight in all sizes (0.4 to 3.9 g) in *G. maculatus* with an increased protein level in the diet (Figure 1; Tables 4 and 5, Exps. 1 to 4). In experiment 3.1, the protein% values rise from 26 to 56%, showing significant differences (P<0.05) in the monthly average growth in weight from 13 to 82%; similar results are seen in Exp. 4.2 for the extruded diet (55% of protein) with a 171% weight increase. In experiments 1, 2.1 and 3.1, diets under 32% protein are associated with a significantly lower growth (P<0.05) than diets with a higher percentage of protein. The high variability in the results of diets with higher protein levels expressed in coefficient of variations from 24 to 61% reveal no significant differences with the diets with lower protein levels, for example Figure 2, Table 4, Exps. 1, 2.2 and 3.2. Water pond monthly mean temperatures fluctuated between 9.4 and 16.7°C and are shown for each experiment in Table 4.

The results also show that the monthly growth percentage depends on fish weight. On a higher weight but lower growing percentage, for pelletized diets between 37

Table 3. Average and standard deviation of proximal analysis of isocaloric pelleted diets with different percentages of fishmeal used in the experiments.

Diet	Experimental				Commercial			
% Fishmeal in diet	**20**	**35**	**51**	**67**	**NS**	**V1**	**With FO**	**Without FO**
Mean % Diet components								
Dry matter	93 ± 5	96 ± 3	92 ± 2	92 ± 1	93 ± 1	99	93	92
Crude protein (N × 6.25)	25 ± 3	31 ± 1	39 ± 6	48 ± 10	56 ± 4	55	63	62
Lipid (Ether extr. Soxhlet)	16 ± 4	22 ± 8	16 ± 4	20 ± 4	17 ± 4	15	21	8
Non-nitrogenated extr. (ENN)	54 ± 5	42 ± 8	36 ± 11	24 ± 11	20 ± 8	18	5	17
Total ash	5 ± 1	4 ± 3	7 ± 3	8 ± 1	13 ± 2	11	11	13
Fibre	1 ± 1	1 ± 1	1 ± 1	1 ± 1	1 ± 0	2	1	1
Gross Energy (MJ kg^{-1})	21.35 ± 0.9	23.13 ± 2.0	21.78 ± 0.9	23.05 ± 0.5	23.15 ± 2.2	21.90	23.80	20.40

NS= Nutra Starter Trow Chile Skretting; V1: Vita caliber 1 Salmofood; FO: Fish Oil.

Table 4. Percentage of average monthly growth in weight and adult feed conversion ratios of *G. maculatus* due to protein and lipid effects in the diet in experiments 1 to 5. Experiments 1 to 4 n= 3, experiment 5 n= 4. Significant differences are in the monthly weight growth% of treatment<0.05 (*).

	D	%P	%L	E (MJkg^{-1})	PE/TE	Weight$_i$ (g)	Weight$_f$ (g)	%Growth month	SD	%CV	SGR (%d^{-1})	GF3	FCR	T°C mean
Exp. 1 month 1	1Pel.	27	13	20.25	0.30	0.4	0.51	29 *	5.0	16	0.7			
	2Pel.	32	12	20.86	0.36	0.4	0.61	54	14.0	27	12			
	3Pel.	37	18	22.53	0.38	0.4	0.63	56	18.0	33	1.3			
Exp. 2.1 month 1	1Pel.	27	13	20.25	0.30	1.4	1.5	9	8.0	87	0.1	0.07	2.5	12.0
	2Pel.	32	12	20.86	0.36	1.4	1.8	29	30.0	50	0.3	0.26	3.2	
	3Pel.	37	18	22.53	0.38	1.4	2.1	50	45.0	25	0.6	0.44	1.5	
Exp. 2.2 month 1	1Pel.	27	13	20.25	0.30	3.9	4.4	14 *	3.0	22	0.2	0.15	3.1	13.7
	2Pel.	32	12	20.86	0.36	3.9	4.5	15	4.4	29	0.2	0.18	2.0	
	3Pel.	37	18	22.53	0.38	3.9	4.6	18	2.6	14	0.2	0.21	1.6	
	4Pel.	57	16	22.97	0.58	3.9	4.9	26	6.5	24	0.3	0.29	1.0	
	5Ext.NS	53	20	24.60	0.50	3.9	5.2	34	1.5	4	0.4	0.37	0.7	
Exp. 3.1 month 1	1Pel.	26	21	22.52	0.27	1.6	1.8	13 *	3	23	0.2	0.13	4.0	12.0
	2Pel.	32	26	24.71	0.30	1.1	1.5	43	14	33	0.8	0.31	2.0	
	3Pel.	45	20	22,61	0.47	1.4	1.9	38	18	49	1.0	0.33	2.0	
	4Pel.	56	19	22.90	0.57	1.2	2.1	82 *	13	16	1.4	0.61	1.0	
	5Ext.V1	58	14	21.55	0.63	1.8	2.2	23	17	74	0.4	0.23	2.0	
Exp. 3.2 month 2	1Pel.	26	21	22.52	0.27	1.4	1.9	36	7	18	0.7	0.42	2.8	9.4
	2Pel.	32	26	24.71	0.30	1.5	2.1	38	7	18	1.1	0.48	1.1	
	3Pel.	45	20	22.61	0.47	1.9	2.5	32	12	37	0.7	0.42	0.9	
	4Pel.	56	19	22.90	0.57	1.8	2.8	57	35	61	1.1	0.68	1.5	
	5Ext.V1	58	14	21.55	0.63	2.0	3.3	70	32	45	1.7	0.81	0.7	
Exp. 4.1 month 1	1Pel.	20	17	21.31	0.22	0.5	1.6	213	31	14	3.8	0.75	1.0	16.7
	2Pel.	30	27	23.82	0.29	0.5	1.3	160	35	22	3.2	0.59	1.3	
	3Pel.	43	14	21.06	0.48	0.5	1.5	193	31	16	3.6	0.70	1.1	
	4Pel.	40	26	23.81	0.40	0.5	1.5	193	31	16	3.6	0.70	1.2	
	5Ext.V1	55	15	21.90	0.59	0.5	1.3	167	83	50	3.2	0.59	1.5	

D= Diet; %P= % Protein; %L= % Lipid; E= Energy; PE/TE= Protein Energy/Total Energy; SD= Standard Deviation; %CV= % Coefficient of Variation; SGR= Standard Growth Rate; GF3= Growth Factor 3; FCR= Feed Conversion Ratio.

Table 4. Continued...

	D	%P	%L	E (MJkg^{-1})	PE/TE	Weight$_i$ (g)	Weight$_f$ (g)	%Growth month	SD	%CV	SGR (%d^{-1})	GF3	FCR	T°C mean
Exp. 4.2 month 2	1Pel	20	17	21.31	0.22	1.6	1.9	24 *	10	42	0.7	0.15	2.2	15.6
	2Pel.	30	27	23.82	0.29	1.3	2.0	58	22	38	1.5	0.36	1.0	
	3Pel.	43	14	21.06	0.48	1.5	2.4	67	21	31	1.7	0.41	0.8	
	4Pel.	40	26	23.81	0.40	1.5	2.6	80 *	16	20	1.9	0.49	0.7	
	5Ext.V1	55	15	21.90	0.59	1.3	3.0	171 *	41	24	3.3	0.75	0.5	
Exp. 5 month 1	Ext.	63	8	20.40	0.71	0.6	1.0	67	24	35	1.7	0.4	1.8	13.4
	Ext.	63	21	23.84	0.62	0.6	1.1	105	25	24	2.4	0.5	1.3	
month 2	Ext.	63	8	20.40	0.71	1.0	1.4	51	26	51	1.3	0.3	1.8	12.5
	Ext.	63	21	23.84	0.62	1.1	1.8	71 *	16	22	1.8	0.5	1.3	

D= Diet; %P= % Protein; %L= % Lipid; E= Energy; PE/TE= Protein Energy/Total Energy; SD= Standard Deviation; %CV= % Coefficient of Variation; SGR= Standard Growth Rate; GF3= Growth Factor 3; FCR= Feed Conversion Ratio.

Table 5. Equations and correlation coefficients of the logarithmic relationships in protein percentage and protein energy/total energy diets (PE/TE) and monthly weight growth rate for different average weights of *G. maculatus.*

Weight	Relation	Relation
(g)	% protein - %monthly growth	PE/TE - %monthly growth n
0.4	Y= 87.422 ln(x) – 25.930 r^2= 0.84	Y= 120.040 ln(x) + 174.10 r^2= 0.99 9
0.5		Y= -10.440 ln(x) + 178.26 r^2= 0.03 15
1.3	Y= 72.788 ln(x) – 220.870 r^2= 0.76	Y= 65.372 ln(x) + 106.60 r^2= 0.67 15
1.4	Y= 129.780 ln(x) – 419.370r^2= 0.99	Y= 157.250 ln(x) + 196.71 r^2= 0.90 9
1.5	Y= 65.001 ln(x) – 166.770 r^2= 0.89	Y= 59.777 ln(x) + 123.04 r^2= 0.75 15
1.7	Y= 20.134 ln(x) - 32.517 r^2= 0.39	Y= 19.112 ln(x) + 36.11 r^2= 0.96 15
3.9	Y= 16.813 ln(x) - 42.344 r^2= 0.98	Y= 18.337 ln(x) + 58.31 r^2= 0.36 15

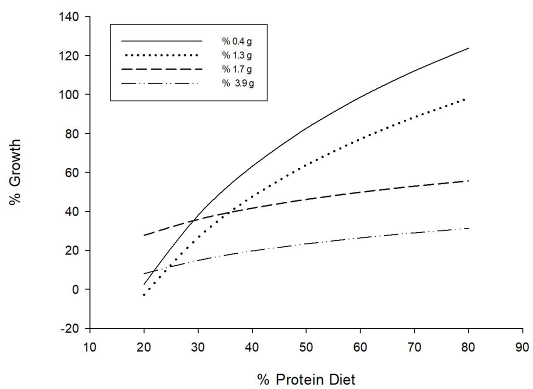

Figure 1. Logarithmic trend ratio of the percentage of protein in the diets and monthly growth rate for different weights of *G. maculatus*, with points estimated from Table 5 equations.

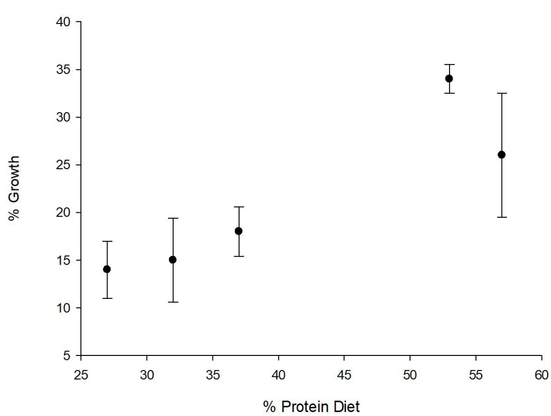

Figure 2. Growth percentage of *G. maculatus* pelletized fed diets containing different protein levels in Experiment 2.2. Extruded diet 53% protein.

and 57% of protein, there was maximum growth from 193 to 26% monthly in newly metamorphosed juveniles from 0.4 to 3.9 g adults. Higher growth for different weights is associated with lower feed conversion ratios, fluctuating between 0.7 and 1.5, in a protein energy/total energy (PE/TE) ratio of 0.38 to 0.58 and diet energy from 21.06 to 23.81 MJ kg^{-1} (Table 4).

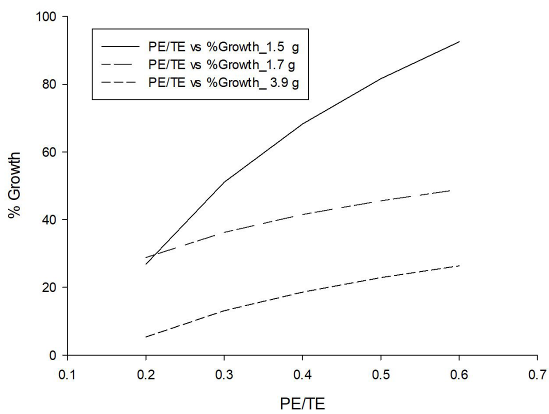

Figure 3. Logarithmic relationship trend between protein energy/total energy (PE/TE) diet and percentage monthly growth rate of three weights of *G. maculatus* (1.5, 1.7 and 3.9 g), with estimated points from Table 5 equations.

From an energetic approach, the results also show that there is a tendency to a linear trend between the energy of the diet (MJ kg^{-1}) and the % of weight increase (*e.g.* Exp. 2.1: Y = 16.831 X - 327.84 R^2 = 0.9337). The logarithmic relationship (protein energy/total energy of the diet) as it increases from 0.2 to 0.6 increases the monthly weight growth percentage of *G. maculatus* (Figure 3, Tables 4 and 5, Exps. 1, 3.1, 3.2 and 4.1). Lower weight adult fishes with a low PE/TE ratio of 0.3 in their diet have a low monthly growth rate (0.4 g: 30% to 1.4 g: 7%) and with a high ratio (0.6), a higher growth rate (116 to 73%) (Table 5). Higher weight adult fishes and a low PE/TE diet ratio of 0.2 also show low monthly growth (1.7 g: 29% to 3.9 g: 5%) and with a 0.6 ratio show the strongest growth (49 and 26%, Figure 3).

The results show that a higher % protein in the diet or growth in weight lower feed conversion ratio. The feed conversion ratio in the pelleted diets vary from 0.7 to 1.5 and in the extruded reaches up to 0.5 (Figures 4 and 5, Exps. 2.1, 2.2, 3.2 and 4.2).

When a commercial extruded salmon diet is compared with an experimental pelleted diet, in three out of four experiments, *G. maculatus* growth was greater on the extruded diet with a lower feed conversion ratio (Table 4).

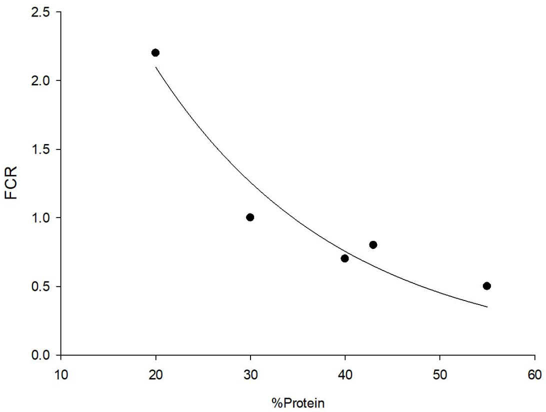

Figure 4. Potential negative trend of protein percentage in diets v/s feed conversion ratio (FCR) in Experiment 4.2. $Y= 134.8\ X^{-1.4}$ $R^2 = 0.955$.

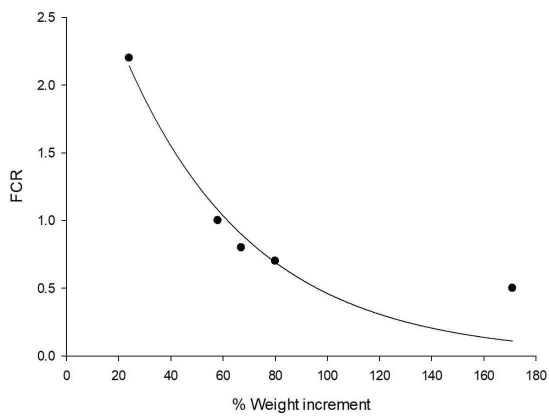

Figure 5. Potential negative trend in the monthly percentage increase in weight v/s feed conversion ratio (FCR) diets in Experiment 4.2. $Y= 22.9X^{-0.7702}$ $R^2= 0.96$.

Growth differences between both types of diet vary between 8 and 91% (Exps. 2.2 and 4.2), even with the significant differences in experiment 4.2 (*P*<0.05). This trend of higher growth and smaller feed conversion ratio in the extruded diet happens when the extruded diet in experiment 2.2 (Figure 2) is higher than the pelleted one (24.60 v/s 22.97 MJ kg^{-1}) with a lower protein level (53 v/s 57%), such as in experiment 4.2, where extruded diet had lower energy levels (21.90 v/s 23.81 MJ kg^{-1}) but higher protein levels (55 v/s 40%). Only in experiment 4.1 did the pelleted diet generate a higher average monthly growth (213%) then the extruded one (167%); however, no significant differences were found (*P*>0.05) and the feed conversion ratio was lower (1.1 v/s 1.5). Pelleted and extruded diets have similar energy values (21.06 and 21.90 MJ kg^{-1}), unless the pelletized diet is less compared to the extruded one, where the PE/TE value (0.48<0.59) and protein level is 43%<55%. Extruded feed conversion ratios are less for the pelleted diets (0.7 v/s 1.0; 0.7 v/s 1.5 and 0.5 v/s 0.7) and PE/TE values between the same pelleted and extruded diets are either higher or lower (0.50<0.58; 0.63>0.57 and 0.59>0.40). These results suggest that: a) A pelleted diet with a varying PE/TE ratio does not increase growth or a lower feed conversion ratio than an extruded diet with similar protein and lipid levels. b) There is an effect of the food preparation process where food extrusion affects growth and feed conversion ratios.

In the experiments, fish growth is affected by diseases causing mortality and sexual maturity. A monthly mean mortality of 5 to 45% is higher than usual in fish farming and some replicates are affected by diseases increasing mortality and decreasing fish number per pond; *e.g.*, 48% in the extruded diet of Exp. 4.2, due to flavobacteriosis. The fast initial sexual maturity of the specimens prevented continuing the experiments for more than 30 to 60 days (Exps. 2.1, 3.1) because the fish start to mature, returning from the sea as crystalline juvenile and metamorphosing into adults when returning to fresh water. The monthly mean sexual maturity of the specimens ranged between 0 and 85% (Exps. 2.1, 2.2, 3.1 and 5). No direct relationship between mortality and sexual maturation was observed in the experiments and there were no significant differences (*P*>0.05) between fish maturity percentages with different protein levels in the diet (Exps. 2.2 and 3.1). However, there is a trend ($Y=0.693X + 39.723$, $R^2=0.8341$) where increasing the level of dietary protein increases sexual maturity; *e.g.*, from 30 to 60% protein increased sexual maturity from 60 to 80% in Exp. 3.1.

3.2. Effect of two level lipid content at a similar protein level in the extruded diet on the increase in weight of G. maculatus

In 0.6 g specimens of *G. maculatus* fed a 63% protein and an extruded commercial diet with two different lipid levels, when increased from 8 to 21%, (20.40 to 23.84 MJ kg^{-1}) and ratio PE/TE 0.71 to 0.62, the first month weight increase ranged from 67 to 105%. In the second month, in 1.1 g fish, from 51 to 71%, there were significant differences in growth (*P*<0.05) between the lipid levels (Table 4 Exp. 5). The fish, as they grow from one month to the next, slow down their growth. Growing fish had a slower weight growth rate, as shown in the second month growth percentages.

The diet composed of 63% protein and 21% lipid has a higher energy content (23.84 MJ kg^{-1}), a higher growth and the best feed conversion ratio (1.3) compared to the lower lipid diet (8%). This result agrees with the higher increases in weight (105 and 71%) and lower variability (%CV = 24 and 22) in month 1 and 2 (Table 4 Exp. 5). In these two diets, a 21% monthly mortality was observed, with 100% of fishes mature and with no significant differences between the diets (*P*>0.05).

3.3. Minimum protein level in a diet for maximum adult growth in G. maculatus

The results show that maximal *G. maculatus* adult growth is obtained with a diet containing a minimum of 37% crude protein, with 40% the optimal value. With protein above this level, the growth percentage increases, reaching a maximum between 50 and 60% (Figure 6).

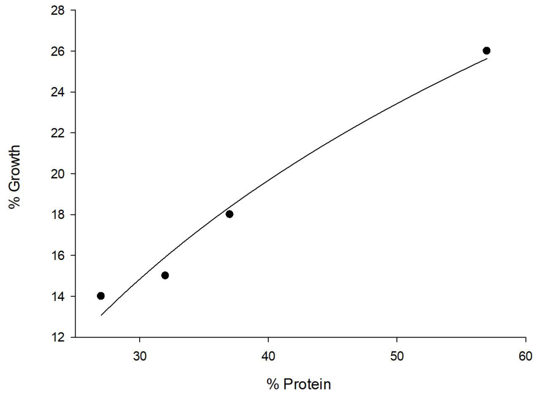

Figure 6. Logarithmic ratio of pelleted protein diet percentage versus monthly growth percentage in *G. maculatus* in Experiment 2.2. Y= 16.81 ln (X) – 42.34 R^2= 0.977.

3.4. Ratio of protein energy vs. total diet energy

The highest monthly percentage growth (82%) is observed for the 0.57 PE/TE ratio, 22.90 MJ kg^{-1}, 56% protein and 19% lipid pelleted diet (Table 4 Exp. 3.1). The highest *G. maculatus* adult growth was obtained on the 0.38 to 0.58 PE/TE ratio, 22.53 to 23.81 MJ kg^{-1} energy level, 37 to 57% protein level and 16 to 26% lipid level pelletized diet. The extruded diet was associated with higher growth rates than the pelleted diets and the best results (171%) were obtained for the 0.59 PE/TE ratio, 21.90 MJ kg^{-1} energy level, 55% protein level and 15% lipid level (Exp. 4.2). In this diet, the PE/TE ratio was in the 0.50 to 0.63 range, with energy values from 21.55 to 24.60 MJ kg^{-1}, protein levels from 53 to 63% and lipid levels from 14 to 21%. The *G. maculatus* protein retention efficiency (PPV) decreases as the protein increases in the diet. The ratio is expressed in the equation: PPV = 87.293 – 1.160 (PE/TE * 100) and predicts a 41% PPV at 10°C when the PE/TE ratio is optimal (0.40).

4. Discussion

4.1. Protein level affects growth

Protein requirements for optimal growth (Garling Junior and Wilson, 1976; NRC, 2011) show a general trend such that at a higher protein level, a higher growth is obtained, reaching an asymptote when the optimal level is exceeded (Shi et al., 1988). This pattern is consistent with the 2.2, 3.1 and 4.2 experimental results (Table 4, Figures 1 and 7), where the optimum is 37% for 0.4 g samples. Protein levels below 30% generate significantly lower growth (P<0.05). Between 50 and 60% protein, higher growth and lower feed conversion ratios are observed (0.5 to 0.7). This value is similar to other carnivorous fish species (Cho and Kaushik, 1990; Tibaldi et al., 1996; Vergara et al., 1996b; Lupatsch et al., 2001; Refstie et al., 2001; Lee and Kim, 2001; Nordgarden et al., 2002; Schuchardt et al., 2008; Jiang et al., 2016).

The protein energy/total energy ratio in a diet is an indicator used to estimate the protein level in fishes (Lee and Putnam, 1973; Santinha et al., 1999; Skalli et al., 2004, Rahimnejad et al., 2015; Jiang et al., 2016). In *G. maculatus* adults, it corresponds to 0.57 and 0.59, reaching the highest growth in extruded and pelletized diets, indicating that a high energy input from protein is required to express their maximum growth. These values are higher than for other species; therefore, growth outcomes with protein levels over 30% are similar to rainbow trout (*Oncorhynchus mykiss*) and arctic char (*Salvelinus alpinus*), requiring diets with an energy ratio of at least 0.35 to retain good growth rates (Jobling and Wandsvik, 1983). In *G. maculatus,* the protein retention efficiency ratio (PPV) decreases with increased protein content in the diet, at 41% PPV when the PE/TE ratio is optimum (0.40) at 10ºC. Similar values were obtained for rainbow trout at 12ºC within the optimum value range between 30 to 40%. Thus, when the diet is high in protein (PE/TE= 0.70) it is poorly utilized for growth (Wandsvik and Jobling, 1982, Wang et al., 2016).

4.2. Lipid and energy affect growth

Increased lipid and energy in the diet allows a protein saving effect or sparing effect (Vergara et al., 1996a; Weatherup et al., 1997; Company et al., 1999; Chatzifotis et al., 2010, Ding et al., 2010., Xu et al., 2015), as well increasing the growth rate and reducing the feed conversion ratio in *G. maculatus* (Exp. 5). When an isoproteic extruded diet is compared (63%) with two lipid and energy levels, at the highest lipid (21%) and energy (23.84 MJ kg^{-1}) level, a significant difference (P<0.05) is obtained in the second month with the 8% lipid 20.40 MJ kg^{-1} energy diet, with a higher growth rate (71%) and a lower feed conversion ratio (1.3). In this experiment, a monthly high mortality is repeated (21%) and 100% of 0.6 g fish initiate sexual maturity. It is remarkable that in all experiments, *G. maculatus* specimens recently metamorphosed to adults start to mature, therefore decreasing the weight growth rate.

4.3. Food processing technique effect

Usually, high lipid extruded diets are associated with higher growth and lower feed conversion ratios than pelleted diets in *G. maculatus*, with results similar to those found by Aksnes et al. (1997), Peres and Oliva Teles (1999), Vergara et al. (1999), Deng et al. (2011), Shapawi et al. (2014), Jiang et al. (2015) and García-Meilán et al. (2016). This is possibly a food extrusion process effect, which for different species improves the digestibility of protein (Deguara, 1997; Pérez et al., 1997; Sørensen et al., 2002), lipids (Peres and Oliva Teles, 1999; Boujard et al., 2004) and carbohydrate (Jeong et al., 1991). In addition, the extrusion process enables one to add more lipid to the diet and hence more energy. This process seems to lead to less protein (53%) extruded food digestion, but more lipid (20%) and energy (24.60 MJ kg^{-1}), generating a higher growth with a lower feed conversion ratio than pelleted food, and with more similar characteristics (Exp. 2.2: 57% protein, 16% lipid and 22.97 MJ kg^{-1}). Again Exp. 4.2 revealed that an extruded diet is associated with a better growth (171%) and feed conversion ratio (0.5), although pelleted diets exceed lipid and energy levels.

4.4. Methodological considerations of experiments

Food and frequency: Food was given *ad libitum* to avoid limiting growth and to guarantee food access to all fish. We estimated that at three daily feedings, one more than used by Jobling and Wandsvik (1983), satiety is reached. This diet approaches the maximum rate that promotes growth, avoiding the loss of food in the bottom of the pond, altering the estimation of food utilization efficiency.

Size: The small size of *G. maculatus* juveniles at metamorphosis to adults of 0.4 g has an effect on the experimental methodology, given that the adult weight used in experiments varied from 0.4 to 3.9 g, constituting a small biomass that requires an even smaller amount of food. For this reason, a pelletized technique was used to provide the small amounts of food required at different protein levels in the test diets.

Diet formulation and elaboration: An isocaloric formulation was designed for pelleted diets to compare the effect of protein level on *G. maculatus* growth. However, the pelleting technique does not allow for the regulation of a high per cent of lipid, leading to variability in amount and consequently in energy.

Wild populations and weight growth variability: Because the farming technology is new and still developing on an experimental basis in *G. maculatus,* we do not have domestic populations. Therefore, these experiment were undertaken in wild populations captured in their spring return to the estuary. The results in the experiments were affected mainly by mortality and sexual maturation. Monthly mortalities fluctuated in a range between 0 and 48%, possibly to stress, but certainly associated with the various endoparasites that they carry. Additionally, in captivity, they suffer epidemic diseases, such as Ich, flavobacteriosis and Salmon Rickettsial Syndrome (Vega et al., 2013). Disease probably affected growth, but certainly deaths decreased the numbers of specimen in the ponds, affecting weight averages, standard deviations and feed conversion ratios.

G. maculatus is a rapidly maturing fish once it reaches adulthood post-metamorphosis at 0.4 g, something that was not known previously. Therefore, experiments were begun immediately once the fish were adapted to eat pellets. However, the adults began to mature quickly, from 50 to 100% in 30 to 60 days, which affected weight growth rates. Thus, the experiments were short term of 3 to 5 months (Table 1) and only the first month's data growth, one or two was considered. Therefore, the experiments must be repeated to obtain and verify clear results and reveal significant differences between the diets. Weight growth results after the first or second month were not considered. Due to mortality and sexual maturation, they exhibit grater variability that prevents statistically differentiating the effect on protein levels of the diets.

5. Conclusion

The results of this work are the first to demonstrate that *G. maculatus,* as a carnivorous fish, requires high protein, lipid and energy levels in extruded diets to grow faster and generate lower feed conversion ratios.

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