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Growth, survival and the histology of the digestive tract of juvenile *Osteoglossum bicirrhosum* (Cuvier, 1829) fed three diets containing different protein and lipid levels

By M. J. Darias[1], M. A. Gómez[2], S. Tello[2] and E. Gisbert[3]

[1]*Institut de Recherche pour le Développement, Unité Mixte de Recherche Biologie des Organismes et Ecosystèmes Aquatiques (UMR BOREA - MNHN, CNRS-7208, UPMC, UCBN, IRD-207), Montpellier, France;* [2]*Instituto de Investigaciones de la Amazonía Peruana, Programa de Investigación para el Uso y Conservación del Agua y sus Recursos, Iquitos, Peru;* [3]*Instituto de Recerca i Tecnologias Agroalimentàries, Unitat de Cultius Aqüicoles, Sant Carles de la Ràpita, Tarragona, Spain*

Summary

The influence of three commercial diets with different protein and lipid contents (40 : 14, 45 : 8, 48 : 8 protein : lipid – P : L, in % dry weight of diet) on growth performance, survival and the histology of the liver and intestine was analysed in early juveniles of silver arowana, *Osteoglossum bicirrhosum*, reared in captivity. Individuals (initial wet weight–W_i = 1.07 ± 0.04 g; total length–TL = 6.0 ± 0.17 cm) were raised in triplicate (n = 12 fish per tank) at 26.2 ± 0.1°C and fed one of three commercial diets over 60 days. At the end of the trial, survival (90.5 ± 3.3%) and condition factor (0.5 ± 0.02) were not affected by dietary treatments, whereas specific growth rate, TL and *W* gain were lower in animals fed the 40P : 14L diet. Histological analysis revealed a larger accumulation of lipid droplets in the intestines of the group supplied with the 40P : 14L diet, as well as a larger surface of hepatic lipid vacuoles compared to the other treatments, although no signs of steatosis were observed. Juveniles fed the 48P : 8L diet displayed the lowest liver lipid accumulation; however, a protein content higher than 45% did not improve growth performance. The intestine and liver lipid accumulation of individuals fed the 45P : 8L diet might indicate a more balanced lipidic metabolism. Intestine and liver histologies proved to be useful markers to identify the nutritional condition in juveniles of *O. bicirrhosum*, even when differences in nutritional composition were subtle (i.e. protein variation of 3%).

Introduction

Osteoglossum bicirrhosum is one of three South American species belonging to the archaic order Osteoglossiformes, characterized by a bony tongue, large ossified scales and laterally-compressed bodies (Goulding, 1980). Adult natural populations inhabiting the lowland and flooded forests in the Amazon basin are strongly exploited for human consumption, and juveniles for the ornamental fish trade (Moreau and Coomes, 2006; Alcántara et al., 2007). As a consequence, the ornamental exploitation of this species is prohibited in Brazil (Prang, 2008), while also being listed as a vulnerable species in Colombia (Mojica et al., 2012), and threatened in Peru (Moreau and Coomes, 2006, 2007).

After Colombia, Peru is the second largest exporter of ornamental species in the Amazonian countries, *O. bicirrhosum* being the most expensive as well as the second most exported species from Peru (Prang, 2008) and the top species exported in Iquitos, the most populated city in the Peruvian Amazon (Moreau and Coomes, 2007). In addition to the management measures needed to control exploitation of this species, development of its aquaculture could constitute an alternative to satisfy the market demands while alleviating fishing pressures on the natural populations.

Our knowledge of the *O. bicirrhosum* ecology is limited (Duponchelle et al., 2012), however, important for the development of its aquaculture. Several studies on its feeding habits and breeding ecology have been published in Brazil (Chaves et al., 2008; Mascarenhas, 2008; Queiroz, 2008), Colombia (Agudelo-Zamora et al., 2007) and Peru (Torres Del Castillo et al., 2012; Cortegano et al., 2014). A recent Peruvian study has shed some light on its life traits, showing an important variability in the growth capabilities of the species between river basins (Duponchelle et al., 2012). This information is of great importance for aquaculture purposes, since the establishment of an efficient aquaculture program begins by the selection of the best-performing broodstock (i.e. populations presenting high growth rates). In this context, aquaculture research of *O. bicirrhosum* is incipient and most studies have been performed mainly to test growth performance under different rearing systems (ponds: Argumedo, 2005; aquaria and troughs: Ribeyro-Schult et al., 2009, 2014; closed recirculation systems: Hernández et al., 2010). However, more research efforts are still needed to improve the existent rearing protocols and, in particular, to gain insights on the nutritional needs at each developmental stage in order to provide adequate diets to support controlled culture. Knowledge on nutrition at early stages is key for obtaining quality individuals while maximizing growth and survival. To our knowledge no nutritional studies have been performed during the juvenile phase in this species. The present study evaluated the effect of three commercial composed diets, mainly differing in their protein and lipid ratio, on growth, survival and histology of the liver and intestine, as these organs are considered to be nutritional and physiological biomarkers (Gisbert et al., 2008).

Materials and methods

Animal rearing

Early juvenile siblings of *O. bicirrhosum* (1.07 ± 0.04 g wet weight-*W*; 6.0 ± 0.17 cm total length-TL, n = 104) were reared at the facilities of the Instituto de Investigaciones de la Amazonía Peruana (Iquitos, Peru) in nine 60-L tanks (50-L water volume; initial density: 12 fish per tank; 3 replicates) at 26.2 ± 0.1°C, 7.2 ± 0.3 mg L^{-1} dissolved oxygen, 7.2 ± 0.1 pH, 167.5 ± 44.5 μ s^{-1} conductivity and 25% daily water renewal. Water physicochemical conditions were measured at 07.00 h (before water renewal and first feeding of the day) every 10 days in all tanks using a portable multi-parameter water quality meter (pH-CE-DO-ORP-ISE, HQ40d, HACH, Loveland, CO, USA). Three commercial diets (Table 1), mainly differing in their protein and lipid content, were used in triplicate to feed juveniles for 60 days, resulting in diets 40 : 14, 45 : 8, 48 : 8 (protein : lipid – P : L, in % dry weight of diet). Juveniles were fed three times a day at 20% of their biomass. The remaining food and faeces were systematically removed before the next food supply.

Animal sampling and measurement of biological parameters

Measurements of TL and *W* were performed every 10 days in all fish (initial n = 36 per treatment, final n = 30–34 fish per treatment) using a 20 cm ichthyometer and a digital balance Ohaus (Ranger 3000, México, ±0.05 g) in overnight fasted fish. The growth performance parameters were measured at the end of the study:

$$\text{Condition factor (K)} = W_f * 100/TL_f^3,$$

$$\text{Specific growth rate (SGR)} = 100 * (\text{Ln } W_f - \text{Ln } W_i)/t,$$

$$\text{Weight gain (WG, } g) = W_f - W_i,$$

$$\text{Total length gain (TLG, cm)} = TL_f - TL_i,$$

where TL_f and TL_i are final and initial total length, respectively; W_f and W_i are final and initial wet weight, respectively; Ln is the natural log; *t*, is the studied period (in days).

Table 1
Proximal composition (given in % dry weight of diet, nominal values provided by the supplier) of three commercial diets used in a 60-day rearing trial of juveniles of *Osteoglossum bicirrhosum*. DE, digestible energy; P, protein; L, lipid

	Diets		
Ingredients	40P : 14L	45P : 8L	48P : 8L
Protein	40	45	48
Lipids	14	8	8
Fibres	3.5	3	2.5
Calcium	1.5	2	2
Phosphorous	1	1	1
Ash	12	12	12
Moisture	10	10	10
DE (Mcal kg^{-1})	3400	3600	3800

Source: Aquatech®, Naltech, Lima, Peru.

Survival at the end of the experiment was calculated as:

$$\text{Survival } (S, \%) = (100 * N_f/N_i).$$

Histological analysis

At the end of the trial, a total of nine specimens (n = 3 per tank replicate) were analysed in order to evaluate the effects of experimental diets on the histology of the liver and intestine. Each sample was dehydrated in a graded series of ethanol, embedded in paraffin and cut into 10 serial sections (3–5 μm thick). In the liver, sections were separated from each other by 80–100 μm; all sections were stained by Harris' Haematoxylin and Eosin (HE) for general histomorphological observations. Size (*S*) of hepatic and intestinal fat deposits (unstained vacuoles within hepatocytes that corresponded to lipids dissolved during the embedding process of the larva in paraffin) was estimated at ×400 magnification according to the formula: $S = 1/4\ \pi\ a\ b$; where *a* and *b* were the minimum and maximum diameters of the vacuole. Average size of melanomacrophage centres (MMCs) was estimated as the mean value between the minimum and maximum diameters. In addition, the number of hepatocytes and MMCs was also counted in six randomly-chosen fields (800 μm^2 per field) per specimen in order to evaluate the level of compactness of the hepatic parenchyma (Bolla et al., 2011). The number of lipid inclusions was also counted in six randomly-chosen fields (800 μm^2 per field) to evaluate the level of lipid inclusion in the intestinal villi.

Statistical analyses

Survival and growth results were expressed as mean ± standard deviation (SD) and analysed by one-way ANOVA using the JMP IN® software (version 4.0.4; SAS Institute Inc., Cary, NC, USA). The Tukey-Kramer post-hoc test was used when differences between treatments were statistically significant ($P < 0.05$). Measurements on histological slides were based on analysis of six randomly-chosen fields in the liver and intestine by means of an image analysis software package (ANALYSIS™; Soft Imaging Systems GmbH, Münster, Germany) and expressed as mean ± the standard error (SE).

Results

Growth and survival

Growth and survival data obtained at the end of the trial are presented in Table 2. Survival was high and similar in all experimental groups ($P > 0.05$). Final *W* in the 40P : 14L group was significantly lower than in the two other treatments (one-way ANOVA, $P < 0.05$), which presented similar W_f values (one-way ANOVA, $P > 0.05$). TL_f in the 40P : 14L group was also lower than that of the 48P : 8L group (one-way ANOVA, $P < 0.05$). The 45P : 8L group resulted in intermediate TL values that were not significantly different from the other two groups (one-way ANOVA, $P > 0.05$). However, TLG and WG were significantly lower in the 40P : 14L group compared to the other two treatments (one-way ANOVA, $P < 0.05$). The condition factor was similar among the three dietary treatments ($P > 0.05$), whereas the specific

Table 2
Survival and growth of juveniles of *Osteoglossum bicirrhosum* fed three diets containing different protein and lipid levels (given in % dry weight of diet) for 60 days at a temperature of 26.2 ± 0.1°C. Data represent means and standard deviation of three replicate tests for each dietary treatment (n = 36 initial number of fish per treatment). Different superscript letters within rows indicate differences statistically significant (One way ANOVA, $P < 0.05$). K, condition factor; L, lipid; P, protein; S, survival; SGR, specific growth rate; TL_i, initial total length; TL_f, final total length; TLG, total length gain; W_i, initial wet weight; W_f, final wet weight; WG, wet weight gain

	Dietary treatments		
	40P : 14L	45P : 8L	48P : 8L
S (%)	88.89 ± 9.62^a	83.33 ± 0.00^a	94.44 ± 9.62^a
W_i (g)	1.03 ± 0.12^a	1.07 ± 0.03^a	1.11 ± 0.06^a
W_f (g)	4.81 ± 0.58^a	6.41 ± 0.75^{bc}	6.54 ± 0.43^c
WG (g)	3.77 ± 0.46^a	5.34 ± 0.77^{bc}	5.42 ± 0.38^c
TL_i (cm)	6.07 ± 0.08^a	5.91 ± 0.34^a	6.05 ± 0.13^a
TL_f (cm)	9.70 ± 0.57^a	10.93 ± 0.55^{ab}	11.07 ± 0.35^b
TLG (cm)	3.64 ± 0.52^a	5.03 ± 0.25^{bc}	5.01 ± 0.23^c
SGR	2.55 ± 0.05^c	2.98 ± 0.22^{ab}	2.94 ± 0.07^b
K	0.53 ± 0.03^a	0.49 ± 0.03^a	0.48 ± 0.02^a

growth rate (SGR) was higher in the 45P : 8L and 48P : 8L groups than in the 40P : 14L group (one-way ANOVA, $P < 0.05$).

Figure 1 shows the growth of *O. bicirrhosum* juveniles fed the different diets during the 60-day trial period. Fish growth was statistically similar among the three groups during the first 40 days of experiment (one-way ANOVA, $P > 0.05$), although a tendency for lower growth rate in the 40P : 14L group was observed from the first month of rearing. From day 50 of the experiment onward, juveniles from this dietary group showed lower growth than the other experimental groups (one-way ANOVA, $P < 0.05$), W_f being 1.5 times lower at the end of the trial.

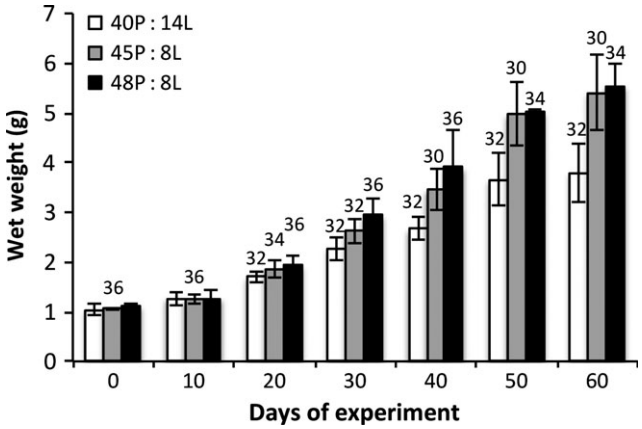

Fig. 1. Growth in wet weight (*W*) of *Osteoglossum bicirrhosum* juveniles reared at 26.2 ± 0.1°C and fed three different compound diets containing variable protein and lipid levels over 60 days. Mean values (data points) and standard deviation (bars) of three replicates for each dietary treatment (40 : 14, 45 : 8 and 48 : 8 protein : lipid – P : L content; dry weight basis, nominal concentrations). Values on top of bars = number of individuals used for *W* measurements per dietary treatment.

Histological analysis

General histological organization of the liver in examined *O. bicirrhosum* specimens consisted of polyhedral hepatocytes typically with central nuclei and arranged in tightly packed anastomosed laminae around veins. The hepatic parenchyma was surrounded by a thin capsule of fibroconnective tissue. The liver structure of *O. bicirrhosum* specimens fed different diets was similar, with differences only found in the level of lipid deposition within hepatocytes (Fig. 2a–c).

In particular, fish fed the 40P : 14L diet had a compact hepatic parenchyma with a large accumulation of lipids within hepatocytes and MMCs scattered in the parenchyma and in the wall of the portal system (Table 3). The volume of hepatic liver inclusions was the highest among the three tested diets, which also translated into a lower number of hepatocytes per field (one-way ANOVA, $P < 0.001$). Fish fed the 45P : 8L and 48P : 8L diets both showed a looser hepatic parenchyma and an increase in size of sinusoids than fish fed the 40P : 14L diet (Fig. 2a–c), as well as smaller volume of fat vacuoles inside the hepatocytes (one-way ANOVA, $P < 0.001$). No differences in the number or size of MMCs were found among dietary treatments (one-way ANOVA, $P > 0.05$).

Contrary to most fish species, the intestine of *O. bicirrhosum* is not the longest portion of the digestive tract, having a similar length to the stomach and pyloric caeca. The histological organization of the intestine in *O. bicirrhosum* specimens was quite uniform throughout its length with only changes in the level of folding of the intestinal epithelium, which was more prominent in the anterior than in the posterior regions. In addition, lipids seemed to be preferentially absorbed in the anterior and mid-regions of the intestine. In brief, the intestinal wall was formed by the serosa, muscularis, submucosa, mucosa and epithelium lined by enterocytes with prominent eosinophilic brush border and abundant large goblet cells. No major histological differences were observed among *O. bicirrhosum* specimens fed different experimental diets (Fig. 2d–f). In particular, the size of lipid inclusions in the intestine was similar among the three tested diets (one-way ANOVA, $P > 0.05$), with values ranging from $11.3 \pm 3.6\ \mu m^2$ in fish fed the 40P : 14L diet, $10.1 \pm 4.4\ \mu m^2$ in fish fed the 45P : 8L diet and $9.4 \pm 3.4\ \mu m^2$ in fish fed the 48P : 8L diet. Although there were no differences in the size of lipid inclusions among groups, fish fed the 40P : 14L diet showed a larger accumulation of lipid droplets than fish fed the 45P : 8L and 48P : 8L diets (47 ± 15 vs 22 ± 11 lipid droplets in 1000 μm^2; one-way ANOVA, $P < 0.05$).

Although livers of *O. bicirrhosum* juveniles fed the 40P : 14L diet did not present steatosis, the protein : lipid proportions and/or composition could affect their lipid metabolism. In contrast, fish fed the 48P : 8L diet displayed livers with the lowest lipid deposition among the three experimental groups. The 45P : 8L diet seemed to be close to the optimal protein : lipid proportion since higher protein and energy content (48P : 8L) did not improve growth.

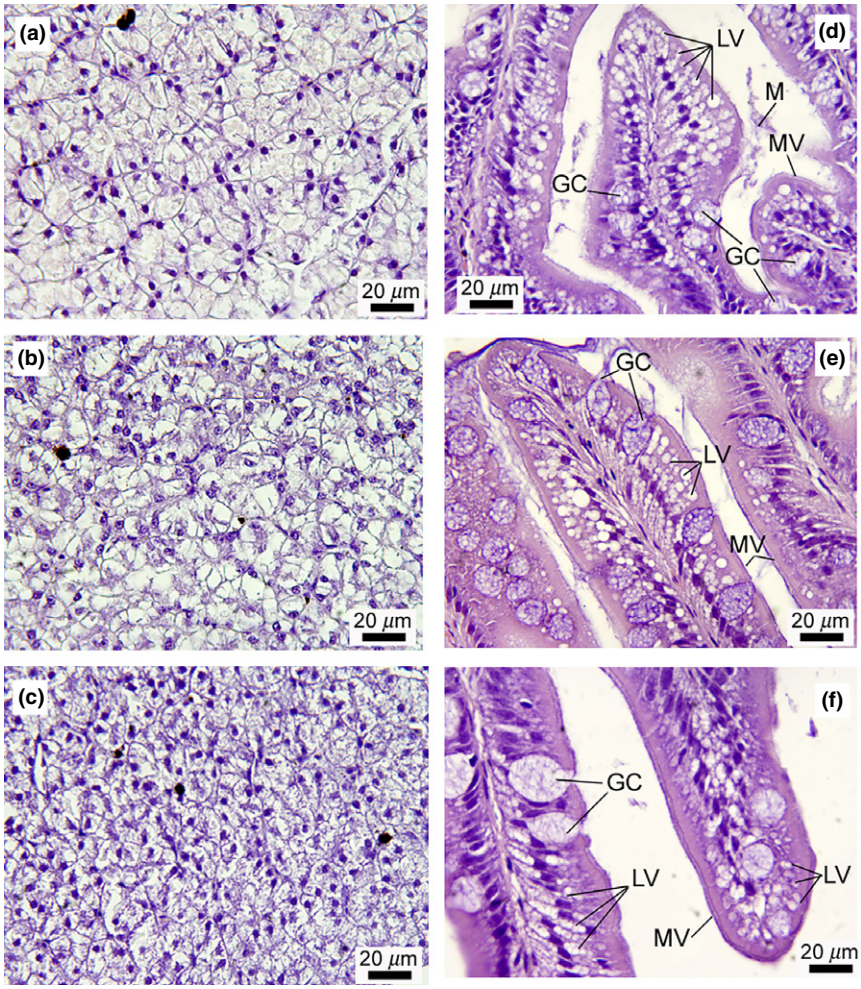

Fig. 2. Longitudinal paraffin sections of the liver (left side) and intestine (right side) *Osteoglossum bicirrhosum* juveniles after a 60-day feeding period (26.2 ± 0.1°C rearing temperature, n = 3 fish per replicate) fed three commercial compound diets, 40 : 14 protein : lipid (upper line), 45 : 5 protein : lipid (middle line) and 48 : 8 protein : lipid (lower line), showing different levels of lipid accumulation. Haematoxilin-eosin staining. GC, goblet cells; LV, lipid vacuoles; MV, microvilli; M, mucus.

Discussion

Adults of *O. bicirrhosum* are opportunistic omnivorous fish with carnivorous preferences, insects being their preferred prey, followed by fish and molluscs (Agudelo-Zamora et al., 2007; Torres Del Castillo et al., 2012). They preferentially forage on various insects (Agudelo-Zamora et al., 2007), which vary greatly in protein (13–77%) and fat content (<5 to >20%) (van Huis et al., 2013; Makkar et al., 2014). Juveniles inhabiting flooded forests additionally feed on algae and micro-crustaceans (Agudelo-Córdoba et al., 2000).

Table 3
Hepatocyte and melanomacrophage centres (MMCs) number in the hepatic parenchyma, surface (S) of lipid vacuoles within hepatocytes and average size of MMCs of *Osteoglossum bicirrhosum* specimens reared at 26.2 ± 0.1°C and fed diets containing different protein (P) and lipid (L) levels (40P : 14L, 45P : 8L, 48P : 8L). Data represent means and standard deviation of three replicate tests for each dietary treatment (n = 3 fish per replicate). Different letters within columns denote statistically significant differences among dietary groups (One-way ANOVA, $P < 0.05$)

Dietary treatments	Hepatocyte number in 100 μm^2	MMCs number in 800 μm^2	*S* of hepatic lipid vacuoles (μm^2)	MMCs size (μm)
40P : 14L	13.5 ± 0.5^a	1.3 ± 0.2	145.7 ± 9.4^a	5.2 ± 0.5
45P : 8L	20.1 ± 0.5^c	1.5 ± 0.2	92.5 ± 8.3^b	5.1 ± 0.4
48P : 8L	17.9 ± 0.7^b	1.3 ± 0.1	54.9 ± 4.3^c	4.5 ± 0.4

The present study aim was to test the nutritional requirements of macronutrients in early *O. bicirrhosum* juveniles under rearing conditions. To our knowledge, this is the first report on the histological description of the liver and intestine of early juveniles of this species. They present a digestive tract morphology with short intestine typically found in fish with chitinolytic activity, and therefore adapted to digest insects (Gutowska et al., 2004). In this particular case, the short intestine is of similar size as the stomach, denoting a carnivorous feeding strategy at this stage.

Results indicate that protein requirements in early *O. bicirrhosum* juveniles are over 40%, and that a 14% lipid content did not spare enough to allow a proper protein deposition for muscle growth. The two other diets contained a lower lipid content (8%); however, higher protein levels (45 and 48%) allowed individuals to show a better growth performance. Differences in growth were related to differences in protein and lipid content and lipid metabolism as reflected by histology of the liver and intestine. The diets tested were

not isocaloric; therefore, differences in performance could also be attributed to differences in the energy content in addition to protein and lipid content.

The histomorphological organization of the liver accurately reflects any physiological disorder originating from a nutritionally unbalanced diet or feed deprivation (Gisbert et al., 2008). Observed dietary effects on the liver may be seen as intra- or extracellular structural changes, of which resorption of glycogen and lipids and changes in mitochondria appearance are the earliest signs. Hepatic energy stores respond sensitively to nutritional changes in deficient diets (Segner et al., 1994). In general, the histological aspect of the liver and intestine was healthy in individuals in all dietary treatments, indicating that none of the evaluated diets were harmful. Similarly, the number and size of MMCs were within the normal range and equal between dietary treatments, indicating no pathological response (e.g. inflammatory lesions) to toxic materials or infectious agents (Agius and Roberts, 2003). Size and type of lipid inclusions in the enterocytes were similar among dietary groups. However, the amount of lipid droplets in the 40P : 14L group was higher than in the other groups, which was correlated with the higher amount of ingested lipids. Fat deposition in both organs reflected some physiological differences, probably originating from a nutritionally unbalanced diet with regards to the nutritional requirements of the species (Segner et al., 1994; Mobin et al., 2000, 2001; Gisbert et al., 2005, 2008). The fattier livers and intestines found in individuals fed the 40P : 14L diet could be related to low dietary protein content. Feeding a low protein diet for a longer period has been shown to induce fatty livers in rats, increased lipogenesis and decreased very-low-density lipoprotein secretion accounting for the hepatic lipid accumulation (Kang et al., 2011). Liver steatosis has also been reported in mice fed diets with low protein and high lipid content (Garcia-Caraballo et al., 2013). In fact, although differences were not significant, SGR tended to be higher in the 45P : 8L group than in the 48P : 8L group, despite the higher energy content of the 48P : 8L diet. This could be associated with the lower liver lipid content observed in histological sections of the 48P : 8L group compared to the 45P : 8L group, as high-protein diets may have anti-lipogenic effects (Santesso et al., 2012). It has been demonstrated in mice that a high protein content prevents lipid accumulation, leading to a lower body weight (Schwarz et al., 2012).

An imbalance in the composition of the diet can modify the capacity to absorb and export lipids through the circulatory system towards the liver to be stored and mobilized when needed (Tso, 1994). In this sense, a similar amount of lipids in liver and intestine has been considered a marker for a balanced metabolism, indicating equilibrium between intestinal lipid absorption and liver lipid storage (Tso, 1994; Tocher, 2003; Boglino et al., 2012). In line with this, the accumulation of lipids in the intestine and liver in individuals fed the 45P : 8L diet showed the most balanced metabolism among the three evaluated diets.

Although the feeding frequency and ration used in the present study were adequate for individuals to grow normally (Argumedo, 2005; Ribeyro-Schult et al., 2009, 2014), growth rates were lower than those reported in the literature for the same species (Argumedo, 2005; Ribeyro-Schult et al., 2009, 2014). However, the different stages of development, rearing conditions, feeding protocols and diet composition used in each study make comparisons difficult. Broodstock origin and quality could also account for great differences in the growth performance at early stages of development, considering that *O. bicirrhosum* presents a long and variable endogenous feeding period that can last circa 30 days (until ca. 38 mm TL) with a mixed feeding period of 10 days (ca. 38–45 mm TL; Yanwirsal, 2013; present study) in captivity or almost 2 months in nature (Aragão, 1984) and that an important variability in the growth capabilities has been observed in nature in individuals of different origin (Duponchelle et al., 2012).

In conclusion, among the three diets tested in this study, the 45P : 8L diet gave the best results in terms of growth and metabolism. Considering that insects are the main source of natural *O. bicirrhosum* food, which present different amino acids and fatty acid composition than fishmeal, commonly the main nutrient of compound diets, further research on their protein and lipid composition is therefore needed to better understand the requirements of these macronutrients. Information regarding the histological organization of the liver and intestinal mucosa in *O. bicirrhosum* can serve as a reference for further nutritional studies on this species.

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Author's address: Maria J. Darias, Institut de Recherche pour le Développement (IRD), UMR 207 BOREA, 911, Avenue Agropolis, BP 64501, F-34394 Montpellier Cedex 5, France.
E-mail: maria.darias@ird.fr