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Larval culture technique and quality control in juveniles of flounder *Paralichthys orbignyanus* (Valenciennes, 1839) in Argentina

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Abstract

Paralichthyds are the most economically important fish in the Southern Atlantic due to their abundance, flesh quality, high market price and increasing fishery demand. In Argentina, the first juvenile mass production of flounder *Paralichthys orbignyanus* in captivity was done in 2002. Morphological description of the different stages of development (yolk sac, drifting, premetamorphic, metamorphic, postmetamorphic larvae and juvenile) are presented, including their duration (days post-hatching), degree-days and feeding schedule. Also, abnormalities in both pigmentation and eye-migration were characterized. Larval developmental stages described in this study for *P. orbignyanus* follow those described for *P. olivaceus*. At 40 days post-hatching (dph) and 798 ºC days, juveniles measured in average 11.6±0.5 mm. Mean survival rate after 142 dph was 49.7%. More than half of the total juveniles obtained (59.1%) were albino, whereas 15.5% were normally pigmented and 13.2% showed abnormal eye migration (dextral or reversed). Development of larval culture methods and quality control of batches will allow the optimization of *P. orbignyanus* juvenile mass production.

Additional key words: eye-migration, flatfish, juvenile abnormalities, fish larval development, fish larval rearing.

Resumen

Técnica de cultivo larvario y control de calidad en juveniles del lenguado *Paralichthys orbignyanus* (Valenciennes, 1839) en Argentina

Los lenguados de la Familia Paralichthydae constituyen un recurso muy importante en el Atlántico Sur debido a su abundancia, calidad de su carne, alto precio en el mercado e incremento del esfuerzo pesquero. En Argentina, la primera producción masiva de juveniles del lenguado *Paralichthys orbignyanus* en cautiverio se realizó con éxito en el año 2002. Se decribe la morfología de los diferentes estadios de desarrollo de esta especie (larvas vitelina, pelágica, premetamórfica, metamórfica, postmetamórfica, y juvenil), relacionándolos con la duración de los mismos (días posteriores a la eclosión), grados días y el esquema alimentario aplicado. Además se caracterizan las anormalidades pigmentarias y aquéllas relacionadas con la migración ocular. Los estadios del desarrollo larvario de *P. orbignyanus* son similares a los descriptos para *P. olivaceus*. A los 40 días posteriores a la eclosión y 798 grados días, se alcanzó el estadio de juvenil con una talla media de 11,6±0,5 mm. La supervivencia promedio obtenida al cabo de 142 días de cultivo fue de 49,7%. Más de la mitad de los juveniles obtenidos (59,1%) fueron albinos, mientras que el 15,5% presentó pigmentación normal. El 13,2% de la camada mostró migración del ojo al lado derecho del cuerpo (individuos dextro). El desarrollo de métodos de cultivo larvario y el control de calidad de las camadas obtenidas, permitirá la optimización de la producción masiva de juveniles de *P. orbignyanus*.

Palabras clave adicionales: anormalidades larvales, cultivo de larvas, desarrollo larvario, migración ocular, pez plano.

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Abbreviations used: DHA (docosahexaenoic acid), dph (days post-hatching), INIDEP (Instituto Nacional de Investigación y Desarrollo Pesquero), NA (*Artemia nauplii*), ppt (parts per thousand), TL (total length).

Introduction

Since 2000, research at the Estación Experimental de Maricultura of the Instituto Nacional de Investigación y Desarrollo Pesquero (INIDEP) is focused on the management and conditioning of *Paralichthys orbignyanus* broodstock (Bambill *et al*., 2006), being the most valuable species of the genus *Paralichthys* due to its abundance, flesh quality and high market price. Also, an increasing fishery demand has been observed with annual catches of 4000 Mg in 2002 and 8000 Mg in 2006 (Anonymous, 2006).

Cerqueira *et al*. (1997) presented preliminary data on induced spawning and larviculture of flounder *P. orbignyanus*, although larvae were unable to feed and total mortality was recorded 9 days post-hatching (dph). Bianchini *et al.* (2005) described general aspects of reproduction, larvae production and on-growing of this species cultured in Brazil, whereas Sampaio *et al.* (2007) evaluated the effects of salinity from fertilization to juvenile settlement, demonstrating the euryhalinity of *P. orbignyanus* even at early stages of development.

The objectives of this paper are i) demonstrate that flounder *P. orbignyanus* is a potential aquaculture candidate under controlled conditions in Argentina, ii) present a preliminary culture technology for juvenile production, from the initial supply of artificially fertilized eggs through the hatchery rearing phase until 142 dph, and iii) assess the juvenile quality through the characterization of larval developmental stages and metamorphic abnormalities.

Material and methods

Broodstock husbandry and egg production

Wild broodstock (N= 17, sex ratio 1 female: 0.7 male) were maintained since 1998 in a circular tank (5 m diameter and 80 cm water depth), connected to a recirculating seawater system (12m^3 h^{-1}) as described by Bambill *et al*. (2006). Body weight ranged from 894 to 4,398 g (mean±SD: 2,571±1,200 g) for females and from 1,068 to 1,948 g (1,528±348.6 g) for males. Total length was from 43.6 to 64.0 cm (57.8±8.4 cm) and from 34.0 to 53.2 cm (49.8±3.8 cm) for females and males, respectively. Broodstock were hand-fed once a day to satiation using each day a different food item (fresh squid *Illex argentinus*, anchovy *Engraulis anchoita* or silverside *Odonthestes argentiniensis*). Salinity ranged from 33 to 36 ppt and pH ranged from 7.30 to 8.40.

To get developing eggs, females were stripped and eggs fertilized with the sperm of 1-2 running males on 13 February 2002 (Bambill *et al*., 2006).

Egg collection and incubation

Egg collection, separation, diameter measurements of egg and oil-drop (data are presented as mean ± SD) were performed according to Radonic *et al*. (2005). Fertilized eggs were disinfected with an iodine solution (1 mL / 40,000 eggs) for 5 min. Then, they were placed in a 70 µm net kept in a 1,500 L tank connected to the recirculating system and provided with enough aeration to avoid hypoxia during embryonic development (Hattori *et al.*, 2004). Fertilization rate (%) was calculated as the number of floating eggs over the total number of fertilized eggs. For the hatching rate, 50 fertilized eggs were placed in each of two 1,000-mL beakers at ambient temperature. After 48 hours the number of live and dead hatched larvae and the remaining eggs were counted. The hatching rate was calculated according to the following equation:

$$\text{Hatching rate (\%)} = \frac{\text{Nº live hatched larvae}}{\text{Nº live hatched larvae} + \text{Nº dead hatched larvae} + \text{Eggs left}} \times 100$$

Larval rearing

Newly hatched larvae were stocked at a density of 10 larvae L^{-1} (Takahashi, 1998; Silva-Arancibia and Castelló-Orvay, 2005) in 1,000 L polycarbonate circular tank by duplicate, being 500 L the volume maintained during the entire trial. The tanks were supplied with a spiral-shaped surface cleaner and aeration. Water exchange was accomplished by the use of plastic hoses (2 m long and 1-2 cm diameter) hanged from one side of the tank, that discharged into the main drain. The inlet hoses were placed inside a circular frame made in plastic, wrapped with a plankton mesh measuring from 200 to 300 µm and 300 to 500 µm during feeding with rotifer, Artemia and weaning. Water exchange in the tanks increased from 20% at 5-10 dph to 300% from 45 dph to the end of the study. Temperature, salinity, pH and larval behaviour were daily recorded. Water temperature in the tanks was influenced by the ambient temperature of the larviculture room.

Microalgae *Nannochloropsis oculata*, cultured according to López (1998, 1999) and López and Oka (2003), were used in the rearing tanks from 1 dph. This method called "green water" is used to maintain environmental stability and prevent rotifer starvation in the tanks (Takahashi, 1998). Microalgal concentration was counted and supplied twice a day to keep a density of 2-5 x 10^6 cells mL^{-1} that allowed the autoreproduction of rotifers in the tank.

The first live prey offered to the fish larvae were rotifers *Brachionus plicatilis* cultured following the technology described by Müller *et al.* (2003). From 2 to 15 dph rotifer density was maintained at 5-10 rotifers mL^{-1} (rot mL^{-1}) and increased to 10-25 rot mL^{-1} until 30 dph. Rotifers in the tanks were assessed 3 times day^{-1} to maintain the required density. Rotifers added to the tanks were previously enriched with 2 x10^6 cells mL^{-1} *N. oculata* for 1 hour.

By 25 dph *Artemia nauplii* (NA) were offered to the fish larvae, previously enriched with a docosahexaenoic acid (DHA) rich oil (Oriental Yeast Industry, Japan) at 0.5 mL/10 L. Artemia density in the rearing tanks was 3 NA L^{-1} at 25 dph, increasing to 150 NA L^{-1} at 30 dph (in 2 doses). From 36 dph, larvae were fed a commercial diet (Love Larva, Hayashikane Sangyo, Ltd., Japan) formulated for the Japanese flounder larvae *Paralichthys olivaceus*, starting the weaning phase. Artemia density was then reduced to attain 50 NA L^{-1} by day 45. Japanese diet of increasing size was given to the juveniles until the fish were 40-50 mm total length at aproximatelly 140-150 dph.

Seed quality production

Stages of development and abnormality frequency (%) were the two parameters used to assess the quality of the juvenile production. Larval developmental stages were identified every 5 days according to Minami (1982) and Kawamura and Hosoya (1997) for the Japanese flounder *P. olivaceus*. Increasing body pigmentation as well as changes in larval behaviour were observed every day. Total length (TL, from the tip of the snout to the posterior margin of the caudal fin) of 20 larvae from each tank, was measured every 5 days until 53 dph. From 54 dph until the end of the study, juveniles were measured every 20 days. Larval body depth (vertical distance across the body from the base of the doral-fin rays to the base of the pelvic fin; Zuñiga and Acuña, 1992) was also recorded. Measurements were registered by means of a profile projector (Nikon V-12B, Japan) and an electronic caliper (Mitutoyo, Japan), and presented as mean ± SD.

Juveniles of 40-50 mm length were assessed for abnormal pigmentation (albinism), incompleted eye-migration and reversed eye migration following Saotome and Aritaki (1988) and Aritaki (1991) for brown sole *Pleuronectes herzensteini.*

The duration of each developmental stage was calculated as dph and degree days (ºd, ºC) calculated as the product of time (estimated when 50% of the larvae were at each particular stage) and the average temperature over a specific growth phase.

Results

Total number of stripped eggs from one female was 116,500 with a 47.5% of fertilized eggs. *P. orbignyanus* eggs were pelagic, transparent and spherical with a unique oil droplet. Egg and oil drop diameters were 850.0±12.0 m and 84.0±2.7 m, respectively. Larvae hatched 24 h after incubation at 18±1ºC. Hatching rate was 19.2% and newly hatched larvae measured 2.17±0.01 mm. Survival from hatching to 142 dph was 49.7%. Salinity and pH were 31-33 ppt and 7.15-8.47 respectively, during the whole larval rearing period.

Larval development

Newly hatched larvae were symmetrical showing a slightly spherical yolk sac with a unique oil droplet that decreased in volume concomitant with larval growth (Fig. 1). Melanophores were spread over the whole body. During this stage the larvae were buoyant remaining near the surface with the head pointing downwards. At 2 dph, the mouth and anus opened and the digestive system became functional. Although larvae did not eat, the eyes were pigmented and prominent. At 4 dph, rotifer mastax were clearly visible inside the gut of 50% of the larvae. Larval pelagic life lasted 30 days and it can be divided into four different stages: i) drifting larva, ii) pre-metamorphic larva, iii) metamorphic larva and iv) post-metamorphic larva. Drifting larva increased the size of the gut and the number of melanophores. Pre-metamorphic stage was characterized by the development of the caudal fin and the notho-cord flexion. Moreover, the first rays of the dorsal fin were only visible at late pre-metamorphic stage. At the

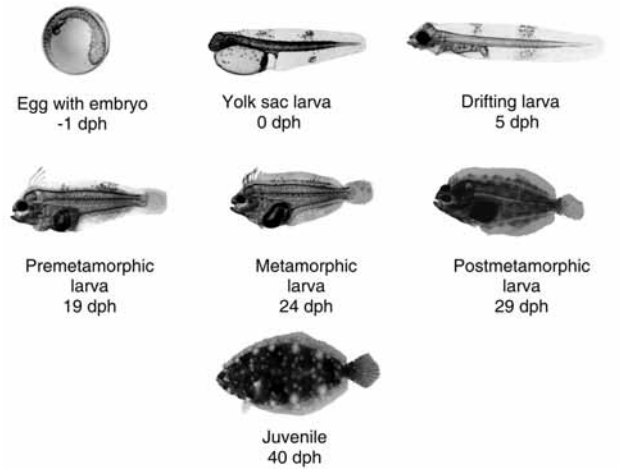

Figure 1. Photomicrographs of larval developmental stages of flounder *Paralichthys orbignyanus* at 20.1ºC. dph: days post hatching.

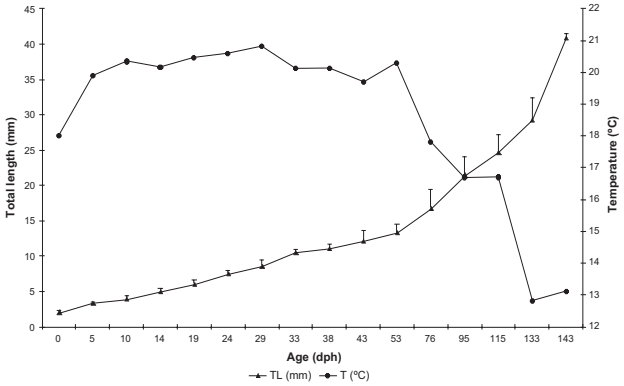

Figure 2. Growth curve versus average water temperature (ºC) during the larviculture of *Paralichthys orbignyanus.* TL: total length; T: temperature.

metamorphic stage the increase in larval body depth was evident and the right eye started to migrate to the left side of the body. The larva was still swimming in the water column. At post-metamorphic stage the right eye was observed at the top of the head while the left eye moved down slightly, becoming the larva asymmetrical. By post-metamorphic stage the right eye has completely migrated to the left side, melanophores increased in number in the ocular side, and the larva moved to the bottom of the tank beginning the Juvenile stage which is characterized by a benthic behaviour. Metamorphosis was completed at 35 dph when the juveniles measured 10.6±0.4 mm. Early juveniles were characterized by the appearance of rays in the pectoral fin by 40 dph. Growth curve of *Paralichthys orbignyanus* larvae and juveniles related to ambient water temperature during larviculture are presented in Fig. 2. Larvae of flounder *P. orbignyanus* could be cultured under experimental conditions at an average temperature of 20.0ºC for the first month. From 53 dph juveniles continued growing, although water temperature decreased during the winter season.

The duration of the developmental stages (expressed as dph and degree days), mean total length and feeding schedule until juvenile stage, are presented in Fig. 3. Larval culture until metamorphosis was completed at 780ºdays. Microalgae and rotifers were the unique live food items offered to the larvae for the first 24 rearing days, and then progressively replaced by Artemia. From 45 dph juveniles fed completely on artifical diet.

Abnormalities in *Paralichthys orbignyanus* juveniles

On 142 dph, 4,088 juveniles were sampled. The frequency of the different types of abnormalities observed during juvenile rearing season 2002, are presented in Table 1 and Fig. 4.

- A: Normal juvenile (Fig. 4A). Complete eye migration from the right to the left side of the body (sinistral). The pigmentation pattern of the ocular side is similar to that of the adults (dark brown with white and dark spots), the blind side is not pigmented.
- B: Malpigmented (albino) juvenile (Fig. 4B): absence of melanophores in the ocular side of the body. Complete right eye migration from the right to the left side of the body.
- C: Incomplete eye migration. The right eye is located at the top of the head. Normal pigmentation pattern as in type A (Fig. 4C).
- D: Incomplete eye migration as in type C and abnormal pigmentation as in type B (Fig. 4D).
- E: Partial eye migration. The right eye cannot be detected from the left side of the body. Juvenile almost symetric. Presence of dark pigmentation on the blind side (ambi-coloration) (Fig. 4E).
- F: Partial eye migration as in type E. Juvenile almost symetric. Both sides of the body without melanophores (Fig. 4F).

The types described above corresponded to juveniles with both eyes on the left side (sinistral) whilst for juveniles with the eyes on the right side (dextral or reversed) the same abnormalities were recorded and indicated with an asterisk A*-F* (Fig. 4A* and 4B*).

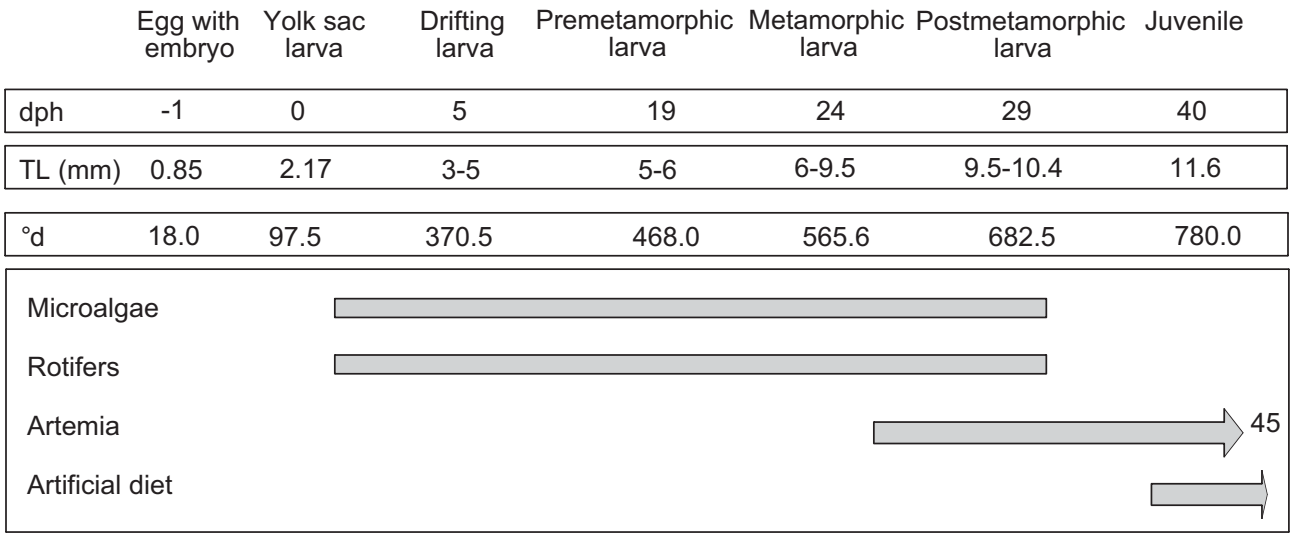

Figure 3. Developmental stages and feeding regime of *Paralichthys orbignyanus* larvae at 20.1ºC. dph: days post hatching. TL: total length. ºd: degree-days.

Discussion

P. orbignyanus eggs are quite similar to eggs of other species of the Family Paralichthydae as cited by Cerqueira *et al.* (1997) and Cerqueira (2005). Egg diameter in these species varies from 0.70 to 1.38 mm (Ahlstrom *et al*., 1984). For *P. orbignyanus*, Bambill *et al.* (2006) reported a mean egg diameter of 818.4±30.1 µm, whereas in the present study the mean diameter was slightly higher (850.0±12.0 m). The differences in egg diameter recorded for flounders can be explained considering that broodstock size, age and genotype, as well as the daily and seasonal feeding rates, can influence the size of the eggs (Bromage, 1994).

Moreover, *P. orbignyanus* larvae show a body length pattern similar to species of the Family Paralichthydae (Cerqueira, 2005). This author reported total lengths at hatching of *P. orbignyanus* larvae ranging from 1.98 to 2.09 mm, when females were hand-stripped. Furthermore, Radonic *et al.* (2007) verified that larvae obtained from natural spawnings were significantly larger (2.34±0.10 mm) than those obtained from hand stripping (2.19±0.10 mm).

Larval developmental stages described in this study for *P. orbignyanus* follow those described for *P. olivaceus* (Okiyama, 1967; Takahashi, 1998), *P. microps* (Silva-Arancibia, 1988) and *P. patagonicus* (Bambill *et al.*, 2000). After 40 dph and 798ºd *P. orbignyanus* fish reach the juvenile stage. Japanese flounder needs 30-44 dph and 600-880ºd to reach the same stage, and earlier than *P.*

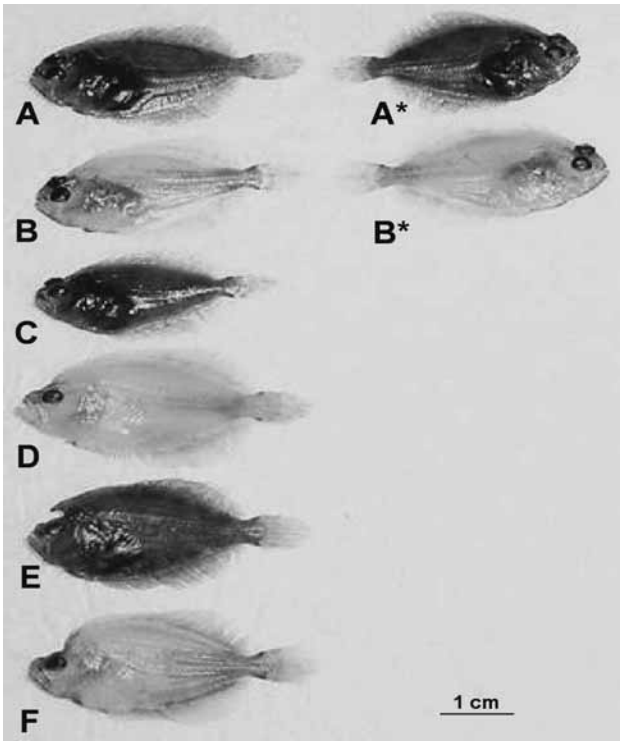

Figure 4. Types of abnormalities observed during 2002 juvenile production of *Paralichthys orbignyanus*. A: normally pigmented juvenile; B: Non pigmented (albino) juvenile; C: pigmented juvenile showing incomplete eye migration; D: albino juvenile showing incomplete eye migration; E: juvenile showing both sides of the body normally pigmented, with no eye migration; F: juvenile showing both sides of the body unpigmented, with no eye migration. A*-B*: dextral juveniles.

Table 1. Abnormality rates found during 2002 juvenile production of *Paralichthys orbignyanus*. A: normally pigmented juvenile; B: Non pigmented (albino) juvenile; C: pigmented juvenile showing incomplete eye migration; D: albino juvenile showing incomplete eye migration; E: juvenile showing both sides of the body normally pigmented, with no eye migration; F: juvenile showing both sides of the body unpigmented, with no eye migration. A*-F*: dextral juveniles

	Normal	Types of abnormalities											Total juveniles
	A	B	C	D	E	F	A*	B*	C*	D*	E*	F*	
Total	635	2,416	19	264	52	155	71	374	2	30	18	52	4,088
(%)	15.5	59.1	0.5	6.5	1.3	3.8	1.7	9.1	0.0	0.7	0.4	1.3	100.0

microps and *P. patagonicus* which complete the metamorphosis at 58-60 dph and 45 dph (810ºd), respectively.

In this study, mass production of juveniles of *P. orbignyanus* has been successfully achieved with almost 50% survival until 142 dph. For the same species, Sampaio *et al.* (2003) cited survivals of 10-15% for larvae obtained from induced spawnings. In the case of *P. adspersus* (Silva, 2001), *P. lethostigma* (Benetti *et al*., 2001) and *P. olivaceus* (Ikenoue and Kafuku, 1992; Watanabe, 1996), survival from hatching to completion of metamorphosis were 25%, 30%, and 60-70%, respectively.

Fish behaviour from 53 dph onwards was clearly influenced by the low temperature in the rearing tanks. Fish were settled on the bottom most of the time showing active swimming when food was offered. Lower water temperature during the weaning period was due to the seasonality in the Southern Hemisphere (winter). Nevertheless, this change does not seem to have influenced the fish growth.

Pigmentation abnormalities are a frequent problem in cultured flatfish lowering considerably their market value. The causes of malpigmentation are not well understood although environmental, nutritional and/or neurological factors have been indicated as the origin of the problem (Seikai, 1998; Venizelos and Benetti, 1999; Bolker and Hill, 2000). According to Takahashi (1998) the occurrence of abnormally pigmented juveniles varied from 1.0 to 29.4% in the case of *P. olivaceus*, whereas for *P. adspersus* (Silva, 2001) and *P. lethostigma* (Benetti *et al*., 2001) have been cited to be 1-5% and 25%, respectively. In the present study, abnormally pigmented juveniles represented more than 50%. Seikai (1998) and Takeuchi (2001) suggested the use of DHA and vitamin A in the enrichment of live prey and early weaning to artificial diets to prevent abnormal pigmentation in flatfish. On the other hand, Takahashi (1998) indicated good husbandry conditions than live prey enrichment in order to prevent these pigmentation disorders.

Reverse asymmetry is also common in reared flatfish, although the causes of this phenomenon are not known. In the left-eyed flounder *P. lethostigma*, abnormal eye migration constituted 0.5-10% in fingerling productions (Benetti *et al.*, 2001). Silva-Arancibia (1988) recorded incomplete eye migration or no migration at all for the dextral *P. microps*. Dextral *P. orbignyanus* juveniles represented 13.2% in this research.

In summary, the results obtained in this study indicate that flounder *P. orbignyanus* could be acclimated and cultured under controlled conditions in Argentina. The larviculture and weanig protocols presented in this study, proved to be appropriate for juvenile mass production at an experimental scale. Stages of development and types of abnormalities were used as indicators of juvenile quality for cultured flounder *P. orbignyanus*.

Research on the causes of *P. orbignyanus* juvenile abnormalities and the effect of water temperature on fish growth, should be carried out in order to optimize juvenile production.

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