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# Hormone-induced ovulation, natural spawning and larviculture of Brazilian flounder *Paralichthys orbignyanus* (Valenciennes, 1839)

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## Abstract

Mature Brazilian flounders *Paralichthys orbignyanus* were captured in coastal southern Brazil and their reproduction in captivity was studied. Brazilian flounder will spawn naturally in captivity when the water temperature is around 23 °C and 14 h of light is provided daily. Females were induced for ovulation and hand stripping using human chorionic gonadotropin, luteinizing hormone-releasing hormone analogue or carp pituitary extract. There was no need to inject males, as running milt was observed during the spawning season. Fertilization and hatching rates were above 80% independent of the hormone used. Notochord length at hatching was 2.18 ± 0.07 mm for larvae hatching from naturally spawned eggs. Larvae were reared in salt water (30–35 g $L^{-1}$) at 24 °C and under continuous illumination. Larviculture was with green water (*Tetraselmis tetrathele* 50 × $10^4$ cells $mL^{-1}$). Rotifers (10–20 ind $mL^{-1}$) were offered as first food 3 days after hatching and gradually replaced by *Artemia* nauplii (0.5–10 ind $mL^{-1}$). Larvae settled to the bottom 20 days after hatching and completed metamorphosis within a week after that. The total length for newly metamorphosed juveniles was 12.9 ± 2.2 mm and the mean survival was 44.8%. The results demonstrate the feasibility of producing Brazilian flounder fingerlings for stock enhancement or grow-out purposes.

**Keywords:** HCG, LHRHa, carp pituitary, reproduction, flounder

## Introduction

The Brazilian flounder, *Paralichthys orbignyanus* (Valenciennes), inhabits estuarine and costal waters of the South Western Atlantic Ocean, from Rio de Janeiro (Brazil) to Mar del Plata (Argentina) (Figueiredo & Menezes 2000). Landings of Brazilian flounder are decreasing, and aquaculture has been suggested as an alternative to increase its production (Sampaio, Bianchini & Cerqueira 2001). The natural spawning season of Brazilian flounder in front of the Lagoa dos Patos estuary (32°1′S–52°0′W) is extended from late spring to summer (Robaldo 2003).

The control of temperature and photoperiod allows the production of fertilized eggs through natural spawning of several marine fish species (Bromage 1995). These include Pleuronectiformes *Psetta maxima* (L.) (Devauchelle, Alexandre, Le Corre & Letty 1988) and *Hippoglossus hippoglossus* (L.) (Bjornsson, Halldorsson, Haux, Norberg & Brown 1998), and some Paralichthyidae, such as *Paralichthys woolmani* (Jordan & Williams) (Benetti 1997) and *Paralichthys lethostigma* (Jordan & Gilbert) (Watanabe, Woolridge & Daniels 2006).

Hormone-induced ovulation through the use of carp pituitary extract (CPE), human chorionic gonadotropin (HCG), luteinizing hormone-releasing hormone analogue (LHRHa) and gonadotropin-releasing hormone analogue (GnRHa) is a common technique widely used to spawn fish in captivity. Several species of Pleuronectiformes respond to

induced ovulation. Smigielski (1975) induced the spawning of wild captured *Paralichthys dentatus* (L.) using CPE, and later Watanabe, Ellis, Ellis and Feeley (1998) successfully obtained fertilized eggs of this species with LHRHa. Human chorionic gonadotropin was used to induce ovulation of *Paralichthys tropicus* (Ginsburg) (Rosas, Arana, Cabrera, Millán & Jory 1999). Gonadotropin-releasing hormone analogue has been used with success to induce the spawning of *Paralichthys lethostigma* (Smith, Mcvey, Jenkins, Denson, Heyward, Sullivan & Berlinsky 1999) and *Solea solea* (L.) (Bertotto, Francescon, Richard, Libertini & Barbaro 2006).

Larviculture of different Paralichthyidae species follows similar protocols. Rotifer is used as first food along with green water. As the larvae grow larger, rotifers are gradually substituted by *Artemia franciscana* (Kellogg) (Bengtson, Gleason & Hossain 1999). Eventually, other live foods are offered to flounder larvae during first feeding, such as trochophore larvae of mollusc and copepods (Benetti, Grabe, Feeley, Stevens, Powel, Leingang & Main 2001).

The first attempts to obtain viable eggs of Brazilian flounder in captivity were performed by Cerqueira, Mioso, Macchiavello and Brugger (1997). In their studies, gravid females were captured in the wild and immediately induced to spawn. More recently, Bambill, Oka, Radonic, López, Müller, Boccanfuso and Bianca (2006) also described induced spawning of Brazilian flounder kept in a recirculating aquaculture system (RAS) for at least 3 years. In both studies, ovulation was successfully induced with HCG, but fish failed to produce fertilized eggs after natural spawning in their holding tanks. However, when fish were hand stripped, artificial fertilization of their gametes resulted in good production of fertilized eggs.

Previous studies on the rearing of Brazilian flounder in captivity have focused on its euryhalinity. Wasielesky, Miranda and Bianchini (1995) were the first to report tolerance of Brazilian flounder to fresh water. Sampaio and Bianchini (2002) determined its isosmotic point to be equivalent to $11\,g\,L^{-1}$, and Sampaio, Freitas, Okamoto, Louzada, Rodrigues and Robaldo (2007) showed that recently metamorphosed juveniles are already able to survive in fresh water.

The objective of this paper is to describe the current techniques used to produce Brazilian flounder fingerlings in captivity, which include natural spawning, induced ovulation and larval husbandry.

## Materials and methods

### Broodstock collection and prophylactic treatment

Brazilian flounder broodstock were captured from the wild. Fish that originated from the commercial fishery in deep waters (20 m) did not survive in the laboratory due to the long time of trawling, approximately 3 h, which caused loss of scales and severely injured the liver and gallbladder. On the other hand, fish captured by artisanal fishermen in shallow waters exhibited good survival after being transferred to the laboratory.

Upon arrival to the maturation facility, Brazilian flounder were treated with a formalin bath in salt water (100 ppm for 30 min) in order to remove external parasites. To control bacterial infections, fish were treated with oxytetracycline (5 ppm) and furazolidone (5 ppm) for 1 week, at a reduced light intensity. The water and the antibiotics were exchanged every day. The body weight and total length of the broodstock used in this work were $1408 \pm 152$ g and $48.5 \pm 1.8$ cm respectively.

### Induced spawning

The efficacy of the following three hormones to induce ovulation in Brazilian flounder was evaluated: HCG ($250\,IU\,kg^{-1}$), LHRHa-des-Gly10[D-Ala6] ($100\,\mu g\,kg^{-1}$) and CPE ($5\,mg\,kg^{-1}$). Wild-captured fish were anaesthetized (benzocaine, 50 ppm) and their total length and weight were recorded and the anaesthetized ovaries were biopsied from the dorsal wall using a 16-gauge hypodermic needle (Harmin & Grim 1992). The follicles sampled were placed in a petridish filled with water equivalent to the isosmotic point ($11\,g\,L^{-1}$) of Brazilian flounder (Sampaio & Bianchini 2002) and were observed under a stereomicroscope equipped with an ocular micrometer in order to measure their diameter.

Preliminary trials showed that Brazilian flounder with oocytes $\geq 400\,\mu m$ will ovulate when induced to spawn with HCG and can be strip spawned. Females received a single intramuscular injection, along the lateral line. The maximum volume of hormone solution injected in each female was 1 mL. Spermiation of males was not induced as running milt was available from all individuals during the trials.

The induced females were kept in 190 L black plastic tanks with controlled temperature ranging from 24 to 25 °C. Females were inspected for ovulation every 6 h, and they were considered to have

 

ovulated when they showed a swollen abdomen. Once they had ovulated, the water volume was reduced to 50 L and benzocaine (50 ppm) was added to the holding tank. Anaesthetized females were hand stripped, and their eggs were fertilized *in vitro* with hand-stripped milt from two males. Only sperm presenting mobile spermatozoa were used in the assays.

Fifteen minutes post fertilization, eggs were washed with running seawater (24 °C) through a 500 μm mesh to remove excess semen and ovarian fluids, and were immediately poured into a graduated cylinder. Fecundity was calculated as the sum of the volume of floating eggs and the eggs deposited on the bottom. A sample of approximately 100 floating eggs was observed under a microscope to determine the fertilization rate, which was assumed to be the percentage of eggs undergoing the first mitotic divisions. A further sample of floating eggs ($n = 36$) was taken and each egg was placed individually on a 96-well plate in order to estimate the hatching rate. The plate was placed in a temperature-controlled room to keep the temperature constant (24 °C).

## Broodstock maintenance and natural spawning

Broodstock were held in black painted, 3000 L, fibreglass maturation tanks, with a bottom area of 5 $m^2$. An external egg collector was attached to the tanks. Each tank was part of an independent RAS comprised of a biological filter (200 L), a ultraviolet lamp (32 $\mu W\,cm^{-2}\,s^{-1}$) and a mechanical filter (5 μm). Temperature was controlled by immersion heaters and air conditioners. Light intensity at the water surface was 0.20 and 0.16 $\mu W\,cm^{-2}$ at the bottom.

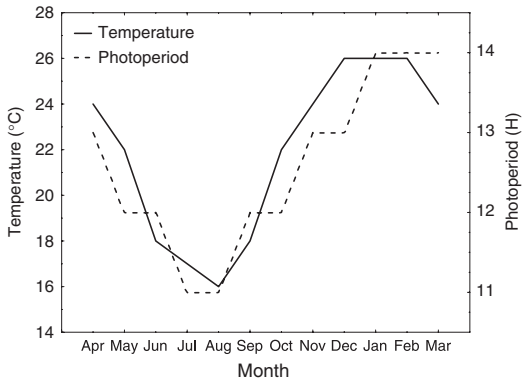


**Figure 1** Photothermal cycle used for conditioning maturation and spawning of Brazilian flounder *Paralichthys orbignyanus* (Valenciennes, 1839).

Brazilian flounder refused to eat inert food during the first weeks of captivity and, consequently, live juvenile fish (treated with a formalin bath for 5 min, followed by a freshwater bath for another 5 min) were offered daily during the first week. Dead fish kept frozen for 48 h were then offered, but the flounder still would not eat inert food. In order to overcome this problem, dead fish were loosely tied to a cotton line and pulled around the tank in order to simulate movement. After a couple of weeks, the fish began to accept inert food and this simulation was discontinued. From that time on, they were fed only on defrosted chopped fish once per day.

Once the Brazilian flounder broodstock was acclimated to captivity, they were stocked, eight per tank, in two maturation tanks (described above). The sex rate was set at one male:one female and stocking density was equal to 1 $kg\,m^{-2}$, equivalent to 2 $kg\,m^{-3}$. Photothermal conditioning was used to induce natural spawning (Fig. 1).

## Live feed production

Microalgae *T. tetrathele* (Kylin) Butch, rotifers *Brachionus plicatilis* Mueller and *A. franciscana* were offered as live feeds to rear larvae up to metamorphosis. Rotifers were batch cultured on microalgae and *Artemia* were hatched according to the protocol of the manufacturer. Rotifers and *Artemia* were washed with abundant salt water before being added to the larviculture tanks.

## Incubation and larviculture

Naturally fertilized eggs were transferred to conical incubators (35 L) with gentle aeration until hatching. Newly hatched larvae were gently transferred to 15 L capacity fibreglass larviculture tanks painted black on the walls and white on the bottom. Constant aeration was provided to keep the oxygen near saturation. Temperature was controlled at 24 °C by immersion heaters equipped with a thermostat. Salinity was kept between 30 and 35 $g\,L^{-1}$ and constant illumination was provided. Dead larvae, faeces and other debris were syphoned out daily.

Larvae were fed exclusively on rotifers until 17 dph (days post hatching); prey density was set from 5 to 20 $mL^{-1}$. Green water was used with the microalgae *T. tetrathele* (500 000 cells $mL^{-1}$) during the entire

growth trial. From day 18 onward, *Artemia* nauplii were also offered, increasing their density from 1 to 10 nauplii $mL^{-1}$ at the end of the experiment, 30 dph.

Larval growth measurements were performed with anaesthetized individuals (MS-222; 50 ppm) under a stereoscopic microscope equipped with an eyepiece micrometer. The standard length of 10 larvae from each tank was measured every 5 days until they began to settle to the bottom of the tank. During the settlement process, growth measurements were discontinued, and they were resumed at the end of the growth trial.

Specific growth rate (SGR) and coefficient of variation (CV) were calculated using the following equations:

$$\begin{aligned} SGR &= (\ln \text{average final length} \\ &\quad - \ln \text{average initial length}) \times \\ &\quad 100/\text{time (days)} \\ CV &= (\text{Standard deviation/mean} \times 100) \end{aligned}$$

## Results and discussion

### Induced spawning

The average diameter of the largest oocyte was approximately equal to 500 µm (Table 1), but oocytes at different stages of development were observed, since small oogonia and vitellogenic oocytes, characterizing Brazilian flounder as a multiple spawner, as other Paralichthyidae species, i.e. *P. dentatus* (Berlinsky, King, Hodson & Sullivan 1997) and *P. lethostigma* (Watanabe *et al.* 2006). Multiple ovulation was observed with all hormones tested, but only the first spawn of each female was used to evaluate the efficacy of the hormones.

Hormonal induction of ovulation for Brazilian flounder was successful with HCG, LHRHa and CPE. The doses used were smaller than those generally used for other Paralichthyidae. Berlinsky *et al.* (1997) induced the ovulation of *P. dentatus* with HCG (total dose for ovulation equal to 500 IU $kg^{-1}$) and CPE (total dose for ovulation equal to 16 mg $kg^{-1}$). Ovulation of *P. tropicus* was obtained with HCG 2000 IU $kg^{-1}$ (Rosas *et al.* 1999). Previous experiments with Brazilian flounder were carried on with higher doses of hormone as well. Bambill *et al.* (2006) achieved good results, inducing the spawning with 1000 IU $kg^{-1}$. It is important to emphasize a possible role of temperature, because in the present experiment Brazilian flounder were maintained at 24 °C, while Bambill *et al.* (2006) maintained their fish at 15 °C.

**Table 1** Spawning induction of Brazilian flounder *Paralichthys orbignyanus* (Valenciennes, 1839) with human chorionic gonadotropin (HCG, 250 IU $kg^{-1}$), luteinizing hormone-releasing hormone analog (LHRHa, 100 µg $kg^{-1}$) and carp pituitary extract (CPE, 5 mg $kg^{-1}$)

| Hormone | *n* | Follicle diameter (µm) | Fertilization rate (%) | Hatching rate (%) |
|---|---|---|---|---|
| HCG | 7 | 542 ± 18 | 81 ± 5 | 90 ± 4 |
| LHRHa | 4 | 484 ± 15 | 90 ± 0 | 82 ± 0 |
| CPE | 4 | 496 ± 21 | 88 ± 7 | 87 ± 6 |

Mean ± standard error.

All the fish injected ovulated within 48 h. Time for the first ovulation was similar, independent of the hormone used, HCG 38 ± 2 h, LHRHa 36 ± 5 h and CPE 40 ± 5 h. The fecundity was also similar among the hormones: 61 000 ± 11 000 eggs $kg^{-1}$ for HCG and 64 000 ± 19 000 eggs $kg^{-1}$ for LHRHa. The fecundity of Brazilian flounder was smaller than that obtained by Bambill *et al.* (2006), with a higher HCG dose at a smaller temperature, which averaged 95 000 eggs $kg^{-1}$ for the first batch of eggs released by three females (calculated from Bambill *et al.* 2006).

Fertilization and hatching rates ranged from 81% to 90%, independent of the hormone used to induce ovulation (Table 1). Conversely, Bambill *et al.* (2006) were able to obtain an average of only 44% fertilized eggs and the hatching rate was in the range of 0% to 30% for Brazilian flounder.

### Natural spawning

Brazilian flounder spontaneously spawned within the first year of captivity. The first spawning was observed 11 months after they were brought to the laboratory. A second spawning was observed the next day, while two other spawning events were observed with a 10-day interval between them. All spawning originated from the same tank, and among them, three were fertilized. No spawnings were observed in the other tank. Spawning was quantified on only 1 day, a total of 120 000 eggs were obtained, but it was not possible to ascertain whether they were all from the same female or not. Spawning events were observed when broodstock were conditioned to long days (14 h of light per day) and elevated temperatures (24 °C). This result contrasts with the appropriate conditions for natural spawning of other Paralichthyidae in captivity, such as *P. woolmani*, which spawns at 20 °C (Benetti 1997) and *P. lethostigma*,

 

which spawns at an even lower temperature of 16 °C (Watanabe *et al.* 2006).

Within a week after the last natural spawning was observed, a massive infection caused by *Amyloodinium* cf. *ocellatum* (Brown) resulted in the loss of all broodstock, a recurrent problem with Brazilian flounder (Abreu, Robaldo, Sampaio, Bianchini & Odebrecht 2005). Before this period, other parasites were eventually observed, mainly the protozoa *Trichodina* sp. and the copepod *Caligus* sp., but no mortalities were associated with their occurrence.

The small size of the maturation tanks might have been an issue to the welfare of Brazilian flounder. Benetti (1997) was able to obtain natural spawns of *P. woolmani* on a 50-tonne tank; however, *P. lethostigma* spawned naturally on 4.8–7-tonne tanks (Watanabe *et al.* 2006).

### Larviculture

The hatching rate of naturally spawned eggs achieved 98%. The notochord length of newly hatched larvae was equal to 2.18 ± 0.07 mm (CV = 3.2%).

The SGR (length) was larger during the endogenous feeding period (7.7% day$^{-1}$); it declined to 3.6% day$^{-1}$ when larvae started feeding, but later on growth was resumed, reaching 6.7% day$^{-1}$ from days 15 to 30 (Fig. 2).

Larvae began to settle to the bottom 20 days after hatching and all larvae completed metamorphosis within a week after that. Their standard length 30 days after hatching was equal to 12.9 ± 2.2 mm, similar to the standard length after metamorphosis of larvae originating from induced spawning (Sampaio *et al.* 2007). The smallest and the largest individuals measured 10.2 and 18.5 mm, respectively, with a CV = 17.1%. Such heterogeneity favours the onset of cannibalism, an important source of mortality during the early larval/juvenile growth stage for predatory fish (Folkvord & Ottera 1993; Kestemont, Jourdan, Houbart, Mélard, Paspatis, Fontaine, Cuvier, Kentouri & Baras 2003). Survival after 30 days was equal to 44.8%, cannibalism was not quantified, but it was possible to observe larger individuals predating on their siblings.

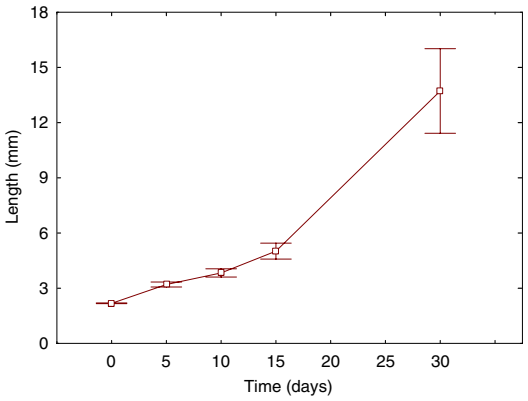


**Figure 2** Growth of Brazilian flounder *Paralichthys orbignyanus* (Valenciennes, 1839) larvae.

## Conclusion

The Brazilian flounder will spawn in captivity either naturally or after hormonal induction of ovulation. The results on natural spawning in this study were not consistent due to protozoan infections and it is likely that the size of the maturation tanks used was smaller than they should be. However, the temperature and photoperiod manipulation applied were effective to obtain naturally fertilized eggs. Human chorionic gonadotropin, LHRHa and CPE successfully induced ovulation, allowing the production of artificially fertilized eggs. Larvae originating from either natural or artificial spawning performed very well under intensive culture, allowing reliable fingerling production.

Induced ovulation and natural spawning of other Pleuronectiformes has been achieved (Berlinsky *et al.* 1997; Bertotto *et al.* 2006), and as a consequence, fingerling production is currently possible for several Paralichthyidae species (Benetti 1997; Bengtson *et al.* 1999). Commercial culture of the Brazilian flounder will be reliable once techniques to obtain good-quality fertilized eggs and healthy fingerlings are developed.

The results shown here demonstrate the feasibility of producing Brazilian flounder fingerlings for stock enhancement or for grow-out purposes.

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