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Effects of salinity on Brazilian flounder *Paralichthys orbignyanus* from fertilization to juvenile settlement

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Abstract

The Brazilian flounder, *Paralichthys orbignyanus*, is a promising candidate for aquaculture, especially due to the euryhalinity demonstrated experimentally for large juveniles (3 g) and sub-adults. Flounder are observed in estuaries and were already reared in fresh and salt water, however little is known with respect of salinity tolerance during their early development. The objective of this work was to evaluate the effects of salinity from fertilization to juvenile settlement. Three experiments were conducted to evaluate the effects of salinity. In trial 1 adult flounder were captured in the wild, transferred to the laboratory and induced to spawn. The gametes were hand striped, split in four samples and fertilized with water at 10, 15, 25, and 35‰. Eggs were considered fertilized when the first cell divisions were observed under the microscope. For the trial 2 newly hatched larvae were reared in four salinities (5, 10, 20, and 30‰) and their growth and survival were observed until metamorphosis. In trial 3 larvae and juvenile of different ages (6, 16, 30, 45, and 60 dah — days after hatching) were evaluated for their tolerance to fresh water. Although the fertilization rate was directly proportional to salinity, hatching was successful only in full salt water. Larvae did not survive in low salinity water (5‰) longer than 6 days, whereas growth was improved when larvae were reared at 20 and 30‰. Young larvae cannot survive in salinities below 4‰, but at 30 dah juvenile presented 100% survival in fresh water. The present findings demonstrate the need for high salinity water (30–35‰) for the successful reproduction and incubation of *P. orbignyanus* eggs. Flounder can be reared successfully at intermediate salinities (20‰) during larviculture, but at lower salinities (5 and 10‰) their survival and growth are impaired. However, immediately after flounder metamorphose into juveniles they survive even in fresh water, demonstrating the strong euryhalinity of this species even at early stages of development.

Keywords: Pleuronectiformes; Reproduction; Egg; Larvae; Fingerling; Development

1. Introduction

The Brazilian flounder *Paralichthys orbignyanus* is an estuarine-dependent species (Chao et al., 1982). It can be found in shallow waters down to 20–30 m depth along the Western Atlantic Ocean coast from Rio de Janeiro (Brazil) to Mar del Plata (Argentina) (Figueiredo and Menezes, 2000).

Although adult flounder are found in estuarine waters, histological analysis showed that females do not ovulate while in the estuary (Silveira et al., 1995). Eggs and larvae of flounder were not found in samples of ichthyoplankton

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surveys in brackish water (Muelbert and Weiss, 1991), suggesting that spawning of *P. orbignyanus* takes place in the ocean.

The euryhalinity of sub-adult *P. orbignyanus* in captivity was demonstrated by Wasielesky et al. (1995) using a wide salinity range from fresh water to 55‰ during 96 h. Survival of juveniles (3–150 g) was not affected by fresh water, but their growth was hampered when comparing to flounder reared in brackish or salt water (Sampaio et al., 2001; Sampaio and Bianchini, 2002).

It is known that the distribution of Pleuronectiformes in the wild is limited by salinity, and such distribution is related to their stage of development (Schreiber, 2001). Larvae of *Paralichthys lethostigma* are more adapted to salt water than brackish water (Moustakas et al., 2004), while juveniles show higher resistance to low salinity water, what is improved as they grow older (Smith et al., 1999). On the contrary, young juveniles of *Paralichthys californicus* tolerate a wider salinity range than older individuals, suggesting that young flounder explore estuaries and coastal lagoons, and as they grow they are more adapted to an oceanic condition (Madon, 2002).

Considering the euryhalinity of juvenile *P. orbignyanus*, this work aimed to evaluate the effects of salinity on earlier stages of development, including gametes, eggs, larvae, and young juveniles. This information should help to develop the culture of Brazilian flounder, because information on the environmental requirements of the early stages of development is scarce.

2. Material and methods

Adult fish used in this work were captured at Cassino Beach (southern Brazil, 32°S) and taken to the Laboratory of Mariculture, Federal University of Rio Grande (FURG).

Water used in the experiments was originated from two sources, fresh water was dechlorinated tap water, and salt water was pumped from the near coast through sand filters. Fresh and salt water were mixed at different proportions in order to achieve the desired salinities. Salinity was measured with a hand refractometer.

2.1. Experiment 1: effects of salinity on fertilization rate of flounder

All adults used in the fertilization trials were acclimated to salinity 35‰ and they were used only once. Females with oocyte diameter larger than 400 μm were induced to spawn with carp pituitary extract (3 mg/kg) and they were maintained individually in tanks with 180 l of sea water at 23 °C. They were ready for stripping 24 to 36 h after the hormone was injected.

Artificial fertilization was performed with the dry method. A pool of semen from three males was split in four portions and activated with water at 10, 15, 25, and 35‰, then they were immediately added to 2 ml of eggs from one female (each female was considered a repetition, four females were used in this trial). Ten minutes after the fertilization the eggs were washed with running water at the same salinity to remove excess of semen and ovarian fluid.

One hour after the eggs were fertilized they were homogenised and a sample of no less than 100 eggs was examined under a microscope to determine the proportion of eggs with developing blastomeres, defined as the fertilization rate. Fertilized eggs were placed in tubes (75 ml) at the same salinity they were fertilized to evaluate the hatching rate, eggs from a single female were placed in three tubes for each salinity. The hatching rates were calculated from the number of fertilized eggs placed in each tube. Thirty newly hatched larvae from each replication were measured.

2.2. Experiment 2: fresh water tolerance of larvae and juvenile flounder

Larvae and juvenile used in the fresh water tolerance trial were sampled from rearing tanks at the desired ages. Fish were carefully counted on individual basis and transferred to their experimental units. For each age, six beakers (1 to 2 l) were used, three were subjected to salinity change and three were used as controls (Table 1). Even though salinity was not reduced in the controls, water was exchanged in order to expose all flounder to the same handling stressors. The water in all experimental units was aerated through air stones.

A stepwise acclimation was applied, with salinity decreasing from 34 to 28‰ within the first 6 h of experiment. During the next 24 h salinity decreased 3‰

Table 1
Age (dah, days after hatching), stage of development, volume of experimental units, number of fish per experimental unit, and length (average±SEM) of larval and juvenile *Paralichthys orbignyanus* exposed to fresh water

Age (dah)	Stage	Volume (l)	Number of fish/unit	Total length (mm)
6	Larvae	1	15	3.44±0.40
16	Larvae	1	15	6.07±0.22
30	Juvenile	2	5	11.01±0.14
45	Juvenile	2	5	19.80±0.86
60	Juvenile	2	5	43.20±2.52

every 6 h, and afterwards the rate of decrease was reduced to 2‰ every 6 h, until the fish were in fresh water, 78 h after the beginning of the exposure. Once fish were in fresh water, they were kept in this condition for 96 h in order to evaluate its effect on their survival.

Food was offered during the experiment, larvae were fed with rotifers (*Brachionus plicatilis*) and *Artemia* nauplii, and juveniles (30 dah, days after hatching) were fed either with *Artemia* or with chopped shrimp (juvenile 45 and 60 dah). Rotifers and *Artemia* were carefully washed with water at the same salinity as the experimental media to avoid an increase of the salinity, green water could not be applied due to the low salinities involved.

2.3. Experiment 3: effects of salinity on growth and survival of flounder larvae through metamorphosis

Artificial fertilization was performed according to the methods described previously, but the hormone used to induce ovulation was human chorionic gonadotropin (HCG, 250 UI/kg) and salinity was 30‰.

Three days after hatching, 6000 larvae (standard length 2.80 ± 0.02 mm, $n = 30$, average ± SEM) were divided in 12 cylinder tanks (10 l) painted black on the walls and white on the bottom. Constant aeration was provided to keep the oxygen at appropriate levels. The tanks were placed in a water bath in order to avoid temperature fluctuation. Four salinities with three repetitions were tested: 5, 10, 20, and 30‰. Salinity was reduced at a rate of 5‰ every 12 h.

Temperature, salinity and the number of dead larvae were registered daily. Dead larvae, feces and other debris were siphoned out every day.

Larvae were fed exclusively on rotifers until 17 dah and prey density was set from 20 to 40/ml. Green water was used with the microalgae *Tetraselmis tetrathele* (500,000 cels/ml) until the use of rotifer was discontinued. From day 18 *Artemia* nauplii were also offered and its density increased from 1 to 10/ml at the end of the experiment. Every morning the remaining prey density was estimated and then brought up to the desired density.

For the growth measurements, fish were sampled from the rearing tanks with a dip net and anaesthetized with MS-222 (50 ppm). They were observed under a stereoscopic microscope, equipped with an eyepiece micrometer, and their standard length was measured. Ten larvae from each replicate tank for each salinity were measured every 5 days. Fish were not returned to their original tanks to avoid handling related mortality, and the number of fish sampled along the experiment was diminished from the initial number to calculate the survival rate.

The growth trial lasted for 18 days, when fish belonging to the same rearing salinity were grouped and the time for settlement was observed at each salinity. The number of settled larvae in each tank was registered every day, until every larvae had settled. Standard length of settled larvae was measured as described above.

2.4. Statistical analyses

All analyses were conducted using the software Statistica 6.0. The effects of salinity on flounder in Experiments 1 and 3 were analysed using one-way ANOVA, followed by the Test of Tukey, while in Experiment 2 survival was compared with Test of student. All analyses were performed with significance level of $P < 0.05$. Percent data were analyzed after they were transformed on arc-sine, but only original data are presented. Results are shown as average ± SEM.

3. Results

3.1. Experiment 1: effects of salinity on fertilization rate of flounder

Fertilized eggs of flounder were observed in the range of 15 to 35‰, but floating eggs were observed only at 35‰, due to their negative buoyancy. As fertilized and unfertilized eggs sank to the bottom at salinities lower than 35‰, fertilization rate could not be established by the floating rate (volume of floating eggs divided by the total volume of eggs spawned). Instead, the cleavage rate was applied. Fertilization rate was directly related to the salinity at which the gametes were exposed. Significantly lower fertilization was observed at 15‰ ($P < 0.05$), but no significant difference was found between salinities 25 and 35‰ ($P > 0.05$) (Fig. 1).

Eggs did not hatch at 15 and 25‰, emerging larvae were observed only at 35‰, with a hatching rate of 55.6 ± 2.4% ($n = 3$). Larvae hatched between 32 and 37 h after the fertilization, and notochord length at hatching was 1.65 ± 0.07 mm ($n = 90$).

3.2. Experiment 2: fresh water tolerance of larvae and juvenile flounder

Independent of age, there was 100% survival during the first 42 h, when the salinity was brought from 34 to 12‰. Within the next 12 h, when the salinity was 8‰, the first dead larvae were observed in the 6 dah group. Sixty hours after the beginning of the experiment,

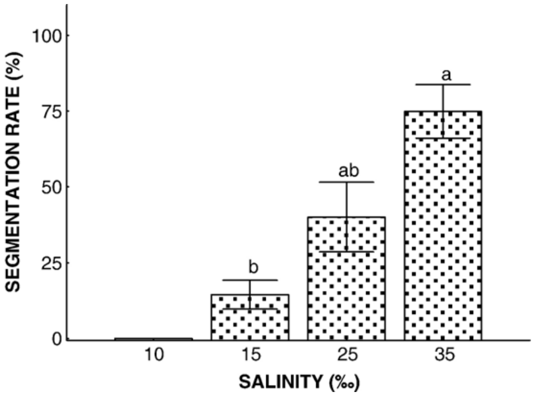

Fig. 1. Effect of salinity on average segmentation rate (±SEM) of *Paralichthys orbignyanus*. Different letters above each bar indicate significant difference among treatments ($P<0.05$). The sample number was 4 at each salinity. Eggs were not fertilized at 10‰.

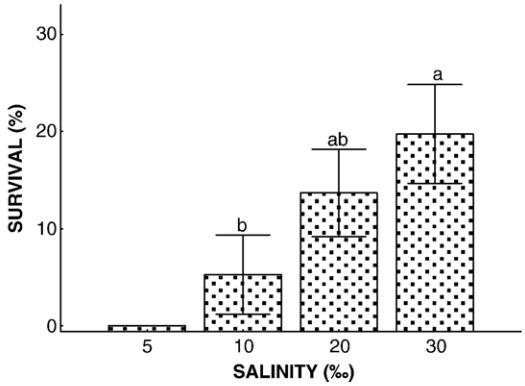

Fig. 2. Survival (average±SEM) of *Paralichthys orbignyanus* larvae reared in different salinities. Different letters above each bar indicate significant difference among treatments ($P<0.05$). The sample number was 3 at each salinity.

salinity was 6‰, and their survival reached 80.0±7.7%. Beyond this point, when salinity declined to 4‰, survival of these larvae was sharply reduced to less than 50% (46.7±10.2%) and the remaining individuals were considered moribund as they were swimming near the bottom of the tank. At the end of the period of salinity reduction (78 h) all larvae in the 6 dah group were dead, but no mortalities were observed in any other age group.

During the 96 h exposure to fresh water, survival was 100% in all juvenile groups, however, survival of the remaining larvae (16 dah group) was 93.3±3.8%.

Among the control groups, dead fish were only observed in the youngest larvae treatment. Their survival was equal to 23.4±7.9% at the end of the salinity reduction period (78 h), decreasing to 20.3±6.6% at the end of the experiment.

3.3. Experiment 3: effects of salinity on growth and survival of flounder larvae through metamorphosis

A high mortality was observed during the first week in all salinities, at this point survival ranged from 9 to 28% among salinity 5 and 30‰. Fortunately, mortality was reduced from this age on, with exception of larvae reared at 5‰, because they were all dead 9 dah.

There was observed a direct relationship between survival and salinity at the end of the experiment. Survival of fish reared at 10‰ was significantly lower ($P<0.05$) when comparing to fish reared at 30‰. However, survival of fish reared at 20‰ was not significantly different ($P>0.05$) from fish reared at salinities immediately below or above (Fig. 2).

Growth was also affected by salinity. Larvae reared at 10‰ were significantly smaller ($P<0.05$) than the others at 13 dah. The same pattern was observed at the end of the rearing trial (18 dah), when larvae reared at 10‰ were significantly smaller ($P<0.05$) than those reared at 20 and 30‰, but there was no significant difference ($P>0.05$) between these last two groups. Larvae reached 6.6±0.1 mm when reared at 30‰ (Fig. 3).

Eye migration was first observed 20 dah and larvae began the settlement process 3 days later in all salinities. At this point, settlement was directly related to the rearing salinity, as 17, 46, and 54% of the larvae were settled, respectively at 10, 20, and 30‰. However, 100% settlement was achieved on day 30 for all groups. Standard length after settlement was 11.2±0.6 mm ($n=30$), 12.8±0.5 mm ($n=30$) and 12.2±1.2 mm ($n=30$), respectively at 10, 20 and 30‰. No mortalities were observed during the settlement period at all salinities tested.

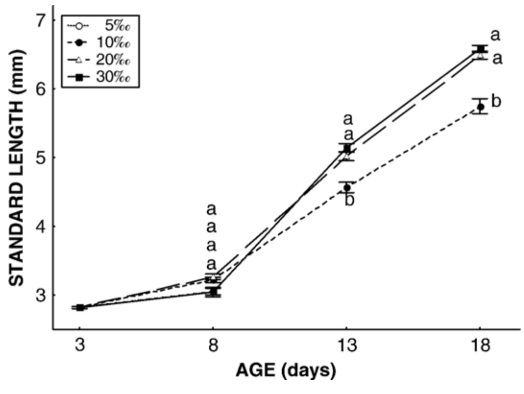

Fig. 3. Growth (average±SEM) of *Paralichthys orbignyanus* larvae reared in different salinities. Different letters within the same age indicate significant difference ($P<0.05$) among treatments (ten larvae were sampled from each replicate tank, as no significant differences of length were observed among larvae from the same salinities ($P>0.05$) they were pooled and the effect of salinity on growth was investigated with $n=30$).

4. Discussion

The effects of salinity on reproduction, incubation, and larviculture of *P. orbignyanus* were determined to be stage/age dependents, and it was observed an increased tolerance to lower salinities as the larvae grew older and metamorphose into juveniles (Table 2).

Among the Pleuronectiformes, there are three families with euryhaline species at different stages of development. In the Pleuronectidae: adult *Platichthys flesus* tolerate fresh water (Arnold-Reed and Balment, 1991) and juvenile *Rhombosolea tapirina* grow well in brackish water, but their survival was reduced at 15‰ (Hart et al., 1996), however growth of juvenile *Colistium nudipinnis* is hampered at 18‰ (Hickman et al., 2002). In the Scophthalmidae: growth of juvenile *Scophthalmus maximus* was improved in brackish water (Gaumet et al., 1995). Besides *P. orbignyanus*, several *Paralichthys* of the Paralichthyidae are euryhaline. *Paralichthys dentatus* and *P. lethostigma* are present in estuaries of the North West Atlantic ocean (Able et al., 1990; Smith et al., 1999) and tolerance to low salinity water has been demonstrated for their larvae and juvenile (Malloy and Targett, 1991; Daniels et al., 1996; Smith et al., 1999). Growth and survival of juvenile *P. californicus* were similar at 8 and 34‰ (Madon, 2002).

In the present study fertilized eggs were observed only at salinities equal to or higher than 15‰, this result matches the findings reported by Pissetti et al. (2002) for the salinity at which spermatozoid of *P. orbignyanus* are activated. These authors did not observe motile spermatozoid at salinities equal or below 10‰, but at 15‰ spermatozoid remained active for at least 10 min. Besides that, osmotic stress was related to the closure of the micropile in eggs of *Fundulus heteroclitus*, blocking the way for the spermatozoid, thus preventing fertilization (Bush and Weis, 1983). Considering that the broodstock used in this work was acclimated to sea water, the exposition of eggs to lower salinities might had induced the closure of the micropile, resulting in progressively lower fertilization rates as the salinity was reduced. Haddy and Pankhurst (2000) demonstrated that water salinity during broodstock acclimation influences the success of reproduction in *Acanthopagrus butcheri*. Other marine fish, such as *Gadus morhua* (Westing and Nissling, 1991) and *R. tapirina* (Hart and Purser, 1995) subjected to artificial reproduction in different salinities also showed an increase in fertilization rates when the salt content in the water was higher.

Table 2
Survival (average ± SEM) of larval and juvenile *Paralichthys orbignyanus* maintained in salt water or exposed to fresh water during 96 h

Age (dah)	Salinity	
	Salt water	Fresh water
6	23.4 ± 7.9[a]	0 ± 0[b]
16	100 ± 0[a]	93.3 ± 3.8[b]
30	100 ± 0[a]	100 ± 0[a]
45	100 ± 0[a]	100 ± 0[a]
60	100 ± 0[a]	100 ± 0[a]

Different letters at each line indicate significant difference ($P < 0.05$) after the Test of Student.

Previous experiments in our laboratory (results not shown) demonstrated that neutral buoyancy salinity for *P. orbignyanus* lies between 26 and 28‰. Eggs of *P. lethostigma* are buoyant at salinities equal or higher than 31‰ (Smith et al., 1999), another Pleuronectiformes, *Hippoglossus stenolepis* also has floating eggs at salinities higher than 30‰ (Liu et al., 1994). In both cases, as it was observed for *P. orbignyanus* in the present experiment, the hatching rates were higher when eggs were incubated at salinities that they could float. Allowing the eggs to sink to the bottom may compromise the water quality as it was suggested by Liu et al. (1994). High hatching rates were observed for eggs of *Mycteroperca rosacea* incubated at salinities below positive buoyancy, but in this case air flow was provided to keep the eggs suspended (Gracia-López et al., 2004).

There is a trend to increase the tolerance to low salinities as the fish grow, which is likely to be related to the development of the gills, as it has been demonstrated to *Lutjanus argentimaculatos* (Estudillo et al., 2000). Gill arches of *P. orbignyanus* begin to differentiate 3 dah, but functional rudimentary filaments and lamellae are observed only 12 dah (Silveira, 1999). Therefore, it is reasonable to suggest that total mortality of young larvae in fresh water could be related to the lack of differentiated gills, as the larvae grow older and the gills were fully developed, survival in fresh water was improved. Skin and intestine are also involved in ionic and osmoregulation, thus the drastic transformations observed in these organs during the metamorphosis of Pleuronectiformes result in an increased tolerance to low salinity waters (Schreiber, 2001). Furthermore, survival of *P. orbignyanus* during the first week of life was low, which coincides with first feeding of their larvae, a critical event for marine fish in general and also verified for *P. dentatus* (Smigielsky, 1975). It is likely that during this period larvae died due to feeding problems and not as a consequence of the salinity stress itself. However, at the end of the growth trial, the lower survival observed for larvae reared at 10‰ was salinity related.

Newly hatched larvae of *Hippoglossus hippoglossus* present higher survival when maintained at 27–32‰ when compared to larvae transferred to lower salinities (Lein et al., 1997). Larvae of *P. lethostigma* were less tolerant to salinity 25 when comparing to 34‰ (Moustakas et al., 2004). However, Daniels et al. (1996) did not find difference on survival of these larvae at salinities 20 and 33‰. In the case of *R. tapirina*, Hart et al. (1996) verified that survival of larvae was improved at salinities 25 and 35‰ when compared to larvae reared at salinity 15‰.

There is no general trend when considering the effect of salinity on growth of euryhaline larvae. Growth of *Pagrus auratus* was similar between 10–35‰ (Fielder et al., 2005), larvae of *Mugil cephalus* grew faster in brackish water in comparison to salt water (Murashige et al., 1991), and larvae of *P. lethostigma* showed a different pattern, higher growth was observed in salt water when compared with 24‰ (Moustakas et al., 2004).

Our findings indicated that the process of settlement was not influenced by salinity. Some environmental factors are known to trigger settlement of different flounder species, but Gavlik and Specker (2004) also did not find salinity to influence settlement in *P. dentatus*. Standard length of newly settled larvae of *P. orbignyanus* was 11–12 mm, similarly the length of other Paralichthyidae in the same developmental stage ranged from 7 to 15 mm (Ahlstrom et al., 1984).

The tolerance of *P. orbignyanus* to low salinity water demonstrated experimentally is in line with its distribution in the natural environment. Adult and sub-adult are present in the estuary of Patos Lagoon, Southern Brazil. They migrate to the ocean for spawning (Silveira et al., 1995), but eggs and larvae can be eventually found in the estuary when the salinity is high (Muelbert and Weiss, 1991). Considering the tolerance of young juveniles to fresh water, it is likely that in the wild juveniles migrate from the coast to the estuary, using it as a nursery ground just like many other teleost (Dando, 1989) and other *Paralichthys* as well (Able et al., 1990; Smith et al., 1999).

Production of marine fish is limited by several factors, but the cost of land by the ocean and environmental permits for open water aquaculture are among the main limiting factors for the development of marine fish culture. Smith et al. (1999) suggested that due to its euryhalinity *P. lethostigma* may be reared inland, in fresh water ponds. The same possibility exists for *P. orbignyanus*, considering that they survive in fresh water since the first month after hatching and the entire grow-out period can be carried out in fresh water. Furthermore, when the euryhalinity of both species is compared, *P. orbignyanus* is more precocious than *P. lethostigma*, because 25 dah juveniles still suffer complete mortality in fresh water (Daniels et al., 1996) and 100% survival will be observed only 220 dah (Smith et al., 1999). Even though growth of *P. orbignyanus* is higher in brackish and salt water (Sampaio et al., 2001; Sampaio and Bianchini, 2002), the lower investment to start a fish farm inland may result in a profitable enterprise.

The present findings demonstrate the need for high salinity water (30–35‰) for the successful reproduction and incubation of *P. orbignyanus* eggs. Flounder can be reared successfully at intermediate salinities (20‰) during larviculture, but at lower salinities (5 and 10‰) their survival and growth are impaired. However, immediately after flounder metamorphose into juveniles they survive even in fresh water, demonstrating the strong euryhalinity of this species even at early stages of development.

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