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Effect of Oxygen Saturation in Water on Reproductive Performances of Pacu *Piaractus brachypomus*

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Abstract

Broodstock pacu *Piaractus brachypomus* as well as their eggs during their embryonic development were exposed to either normoxia (5.5–7.5 mg O_2/L) or hypoxia (2.0–4.5 mg O_2/L) conditions. The plasma concentrations of 11-ketotestosterone in males and estradiol-17β in females, as well as that of their precursor testosterone (T) were significantly ($P < 0.01$) higher in fish maintained under normoxic conditions than in fish exposed to hypoxia. After ovulation and spermiation induced by hormonal treatments, the plasma concentrations of T and 17,20β-dihydroxy-4-pregnen-3-one (17,20βP) significantly ($P < 0.05$) increased in both sexes under both normoxic and hypoxic conditions. The plasma levels of T and 17,20βP achieved under normoxic conditions were higher than the ones recorded under hypoxia, except for those of 17,20βP in males. Males responded positively to the hormonal treatments, and the concentration of spermatozoa was 10.5 ± 0.8 10^9/mL under both oxygen conditions. Hypoxia resulted in significantly lower survival of embryos (17.3 ± 28%) in comparison to normoxic conditions (68.5 ± 25%). Moreover, larval deformities were found when exposed to hypoxia (91.6 ± 6%). During embryonic development of this species 4 mg/L of oxygen is tolerated at 26–27 C without negative impact. We conclude that despite the highly adaptable nature of adult pacu to environmental hypoxia, oxygen concentrations below 4 mg/L severely impacted survival of embryos.

Abiotic factors that may affect egg quality and therefore viability of survival during embryonic stages in teleost fish are physicochemical conditions of the water into which maturing fish (near ovulation or spermiation) are exposed. Brooks et al. (1997) provided evidence for several species of fish where abrupt water temperature changes during final maturation of gametes of females affected egg morphology and subsequent embryonic development. Salinity and pH can also affect quality of eggs produced. However, the effect of oxygen concentrations on gamete maturation in fish has been barely addressed. This effect may be associated with a general response to stress in maturing fish and consequently have a deleterious effect on egg quality. For instance, Campbell et al. (1994) demonstrated that stress during vitellogenesis reduced the

quality of eggs in salmonids. The basis of this susceptibility to stress is unknown since cortisol accumulated in oocytes declined rapidly during embryonic development.

Studies on many species of fish have shown a profound effect of oxygen concentrations on rate of embryonic development, and low lethal levels were established for survival and frequency of deformities (Rombough 1988, 1998). However, less is known on the effects of low oxygen on embryos of Amazonian fishes. This is important to examine, since adults of Amazonian species have developed morphological structures and/or behavioral responses to counter unfavorable oxygen conditions (Braum and Junk 1982; Val and Almeida-Val 1995).

The objective of the present study was to evaluate the reproductive performance of pacu *Piaractus brachypomus* and the embryonic development of their progenies under normoxic or hypoxic conditions. Hormonal treatments were used to induce ovulation and spermiation.

Materials and Methods

The experiment was carried out in October and November 1999 at the field station of the Instituto de Investigaciones de la Amazonia Peruana (IIAP), Iquitos, Peru. Sperm and blood plasma from broodfish were analyzed at the School of Natural Resources, The Ohio State University, Columbus, Ohio, USA.

Mature pacu broodstock were raised in a 1.2-ha pond at IIAP. Average weights (mean ± SD) of males and females were 3.4 ± 0.6 kg and 3.7 ± 0.6 kg, respectively. Spermiating males and robust females were selected. Ova maturity was assessed from catheterized samples of oocytes. Ova were placed in Stockard's solution and the position of the germinal vesicle observed under a microscope. Pairs of pacu were moved into concrete indoor 0.75-m^3 tanks. In each tank, the male was separated from the female by a net. Six and four pairs were used in the first and second trial, respectively. In the first trial, the tanks were not supplied with aeration (hypoxic conditions), whereas intensive aeration was provided in the second trial (normoxic conditions). Fish were allowed to acclimate for 6–12 h in the tanks prior to the hormonal injections. The time span between both trials was 3 d.

Both genders were injected with two doses of Conceptual (Luteinizing Hormone Releasing Hormone analog, LHRHa; Hoeschst Roussel VET, Germany). The concentration of the preparation was 0.0042-mg equivalents of active hormone per mL. Males and females were injected intraperitoneally with 1 mL/kg and 2.6 mL/kg, respectively. The priming dose (50% and 10% in males and females, respectively) was administrated in the morning (1000 h), whereas the resolving dose (50% and 90% in males and females, respectively) was injected at 2200 h. The presence of few eggs at the bottom of the tank, as well as the "knocking" sound produced by the male, were used as signs of female readiness (oviposition). Semen and eggs were collected by stripping. Eggs were weighed, fertilized with pooled semen from three to four males, and incubated in separate vertical (conical) incubators of 60-L capacity. A ring of protective net was installed at the top of the incubators to prevent loss of eggs. In the first trial (hypoxic conditions), aeration was not supplied into the incubators ($N = 3$) and the concentration of O_2 was similar to that measured in the broodfish tank. In the second trial, each batch of eggs was divided and incubated either without aeration ($N = 3$) or with aeration ($N = 6$). Although no aeration was provided into some of the incubators, the concentration of O_2 was higher in those incubators than in incubators in the first trial. This treatment will be then referred to as semi-normoxic conditions.

Sperm concentration was measured after a preliminary fixation in 30% methanol and storage at 2–4 C using a double Neubauer Counting Chamber (VWR Scientific, Cleveland, Ohio, USA) according to Ci-

TABLE 1. *Radioimmunoassay characteristics of steroid hormones in* Piaractus brachypomus.

Characteristics	T	E2	11-kT	17,20βP
Within-assay coefficient of variation (%) (N = 6)	1.7	2.0	2.5	2
Between-assay coefficient of variation (%) (N = 3)	9.2	3.9	6.1	5.9
Accuracy (coefficient of determination)	0.988	0.995	0.984	0.983
Sensitivity (pg/mL)	2	1	2	1
Recovery of extraction (%)	85.8	89.7	91.9	98.6

ereszko and Dabrowski (1993). Sperm was centrifuged at 1,500 *g* for 15 min, seminal plasma collected and stored at −20 C prior to analysis. The osmolality of seminal plasma was measured with an Osmette (Model 5004 Automatic Osmometer, Precision Systems, Natick, Massachusetts, USA). Seminal plasma protein concentrations were determined according to the Bradford (1975) method with Coomassie protein assay (Pierce, Rockford, Illinois, USA) and with bovine serum albumin used as a standard. Lactate dehydrogenase (LDH) activity in the seminal plasma was measured at 20 C according to Vassault (1983) as described by Lahsteiner et al. (1995).

Blood was collected from the caudal vessel of unanesthetized fish prior to the priming injection and at ovulation or spermiation using heparinized syringes. Blood samples were centrifuged at 1,500 *g* for 15 min, and the resulting plasma was stored at −20 C until assays. The plasma concentrations of steroids (testosterone (T), estradiol-17β (E2), 11-ketotestosterone (11-kT), and 17,20β-dihydroxy-4-pregnen-3-one (17,20βP)) were measured by radioimmunoassays similar to those used previously (Ottobre et al. 1989) following ethyl-ether extraction. [1,2,6,7-^{3}H]testosterone (96.5 Ci/mmol) and [2,4,6,7,16,17-^{3}H]estradiol (141 Ci/mmol) were purchased from NEN Life Science Products (Boston, Massachusetts, USA). [^{3}H]11-ketotestosterone and [^{3}H]17,20β-dihydroxy-4-pregnen-3-one were a gift from Dr. C. B. Schreck (Oregon State University, Oregon, USA) and Dr. A. Fostier (INRA, Rennes, France). Unlabelled steroids were purchased from ICN Pharmaceuticals (Costa Mesa, California, USA), Sigma (St. Louis, Missouri, USA), and Steraloids (Wilton, New-Hampshire, USA). The testosterone antiserum was provided by the Institute of Animal Physiology (University of Agriculture and Technology, Olsztyn, Poland), the estradiol-17β antiserum by Dr. R. L. Butcher (West Virginia University, West Virginia, USA), the 11-ketotestosterone antiserum by Dr. D. E. Kime (University of Sheffield, Sheffield, United Kingdom), and the 17,20β-dihydroxy-4-pregnen-3-one antiserum by Dr. A. Fostier. The characteristics of these antisera have been reported previously (Butcher et al. 1974; Kime and Manning 1982; Fostier and Jalabert 1986; Dabrowski et al. 1995). The assay characteristics are shown in Table 1. Extraction blanks were below sensitivity of the assay for examined hormones, and serial dilutions of plasma samples showed parallelism with the standard curve between 25 and 100 μL.

All data are expressed as means ± SE. Homogeneity of variance was verified for all data using Bartlett's test (Dagnelie 1975). Data were subjected to analysis of variance (ANOVA) and subsequent comparison of means by Fisher least significant differences test ($P < 0.05$). Percentage data were arc sin transformed prior to statistical analysis.

Results

In the first trial, the oxygen concentration decreased from 5.2 ± 0.3 mg/L when the fish were stocked in the indoor tanks to 2.2 ± 0.5 mg/L after the first 12 h (hypoxic conditions, Fig. 1). This oxygen concentration was maintained with considerable water flow in the broodstock tanks and then in

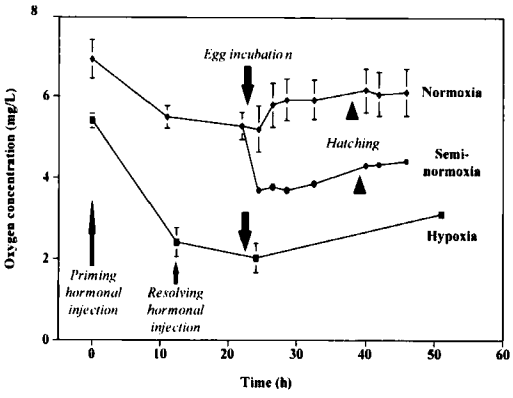

FIGURE 1. *Experimental conditions during two separate trials when pacu broodstock and developing eggs were exposed to either hypoxia (Trial 1) or normoxia (Trial 2). Note in the second trial at the time of initiation of egg incubation (arrow), conditions were differentiated into normoxia (aeration within incubators) and a semi-normoxia (without aeration). Duration is expressed in hours from the time of the priming hormonal injection (see thin arrow). Error bars are not indicated when too small (< 0.2 mg/L). See Materials and Methods for further details.*

the egg incubators. In the second trial, intensive aeration in the broodstock tanks prior to the stocking resulted in an increased oxygen concentration to 7.5 mg/L. At the time of oviposition, the concentration of O_2 was 5.5 ± 0.4 mg/L (Fig. 1). During incubation of eggs, a set of six incubators was maintained with intensive aeration (6.0 ± 1.0 mg O_2/L, normoxic conditions) and the rest were kept non-aerated (4.0 ± 2.0 mg O_2/L, semi-normoxic conditions). In both trials, water temperatures ranged from 26.0 to 27.5 C and the pH was maintained at about 6.2–6.3.

Before the hormonal injections, the plasma concentrations of 11-kT in males (6.0 vs 37.7 ng/mL) and E2 in females (3.9 vs 7.5 ng/mL) as well as that of their precursor, testosterone (0.5 vs 6.8 ng/mL in males and 1.6 vs 6.1 ng/mL in females) were significantly ($P < 0.01$) higher in fish maintained under normoxic conditions than the ones in hypoxia. In contrast, the plasma levels of 17,20βP in males and females were similar regardless of the oxygen concentration (Tables 2, 3). The response patterns of plasma sex steroids to the hormonal treatments were similar in both genders. The concentrations of T and 17,20βP significantly ($P < 0.05$) increased following hormonal treatments in hypoxic and normoxic males and females. The plasma levels of T and 17,20βP achieved under normoxic conditions were higher than the ones recorded under hypoxia, except for those of 17,20βP in males. The plasma concentration of 11-kT in males (37.7 vs 7.5 ng/mL) and E2 in females (7.5 vs 4.6 ng/mL) decreased significantly ($P < 0.01$) after spermiation or ovulation in fish maintained in normoxic conditions, whereas it remained similar in hypoxic fish (Tables 2, 3).

In both trials, oviposition was observed within 8 to 16 h following the resolving dose of the hormone. Two of six females did not ovulate in the first trial (hypoxic conditions), whereas all four females ovu-

TABLE 2. *Plasma sex steroid hormones (mean ± SE) of male* Piaractus brachypomus *under hypoxic and normoxic conditions before and after hormonal injections. Means within the same column with different letters are significantly different (P < 0.01).*

	Plasma sex steroids		
	T (ng/mL)	11-kT (ng/mL)	17,20βP (pg/mL)
Hypoxia ($N = 6$)			
Before injection	0.53 ± 0.10[a]	5.98 ± 1.61[a]	30 ± 10[a]
Spermiation	5.82 ± 0.72[b]	6.29 ± 0.52[a]	116 ± 25[b]
Normoxia ($N = 4$)			
Before injection	6.76 ± 0.98[b]	37.68 ± 5.48[b]	22 ± 9[a]
Spermiation	11.11 ± 1.12[c]	7.46 ± 1.73[a]	106 ± 18[b]

TABLE 3. *Plasma sex steroid hormones (mean ± SE) of female* Piaractus brachypomus *under hypoxic and normoxic conditions before and after hormonal injections. Means within the same column with different letters are significantly different (P < 0.01).*

	Plasma sex steroids		
	T (ng/mL)	E2 (ng/mL)	17,20βP (pg/mL)
Hypoxia			
Before injection (N = 6)	1.58 ± 0.19[a]	3.92 ± 0.23[a]	17 ± 7[a]
Ovulation (N = 4)	11.06 ± 1.80[c]	3.65 ± 0.53[a]	736 ± 253[b]
Normoxia (N = 4)			
Before injection	6.10 ± 1.15[b]	7.46 ± 0.32[b]	45 ± 24[a]
Ovulation	24.14 ± 3.49[d]	4.58 ± 0.75[a]	2,161 ± 865[c]

lated in normoxic conditions (Trial 2). There was no significant difference in the weight of eggs obtained when expressed as percentages of female weight (13.6 ± 4.7%, N = 4 and 13.3 ± 3.2%, N = 4, in Trials 1 and 2, respectively) nor in size of ovulated eggs (1,290 ± 288 and 1,068 ± 100 eggs per gram in Trials 1 and 2, respectively). In both trials, males responded positively to the hormonal injections and the concentration of sperm amounted to 10.5 ± 0.8 × 10^9 spermatozoa per mL independent of the oxygen concentration in the water (Table 4). LDH activity, protein concentration, and osmolality in the seminal plasma did not differ significantly ($P > 0.05$) between fish maintained under hypoxic or normoxic conditions (Table 4).

It was found that embryo survival at 13 h of incubation amounted to 17.3 ± 28 and 68.5 ± 25% in Trials 1 (hypoxia) and 2 (normoxia), respectively. There were no significant differences between normoxic and semi-normoxic conditions in embryo survival. Under hypoxia, three out of four females produced eggs of very low viability 0.5–5.0% survival. The rate of embryonic development was different between the hypoxic and normoxic conditions and the later hatch (20 h) of embryos in the first trial (hypoxia) was associated with much less advanced yolk absorption.

The most significant result of this study was the rate of larval deformities, which was 91 ± 6% under hypoxic conditions (Trial 1) and 1 ± 2% under normoxic conditions (Trial 2). Embryogenesis slowed down considerably when fish were exposed to hypoxia. At approximately 9 h after fertilization, embryos in hypoxic conditions did not complete epiboly, whereas normoxic embryos were already advanced to segmentation (visible myomers) stage. At the time of hatching, embryos in hypoxic conditions were retarded in development and deformities in the posterior part of the body were observed. There were no observable differences between embryo survival and

TABLE 4. *Sperm characteristics (mean ± SE) of* Piaractus brachypomus *broodstock under hypoxic and normoxic conditions. There were no significant differences among trials for any parameter listed (P > 0.05).*

	Trial 1. Hypoxia (N = 6)	Trial 2. Normoxia (N = 4)
Sperm		
Concentration (10^9 × spz/mL)	10.7 ± 1.4	10.1 ± 0.6
Seminal plasma		
LDH (U/L)	20.5 ± 13.5	28.7 ± 15.7
Protein (g/L)	0.099 ± 0.036	0.195 ± 0.038
Osmolality (mOsm/kg)	222 ± 27	265 ± 39

frequency of larval deformities in Trial 2 with or without aeration during egg incubation.

Discussion

Characid fish are well adapted to environmental hypoxia with morphological and behavioral cues (Kramer and McClure 1982; Saint-Paul 1984; Val et al. 1999). In *Piaractus mesopotamicus,* Saint-Paul and Bernardinho (1988) observed that below 0.5-mg O_2/L juveniles were able to use surface water for aquatic respiration. An intense locomotory activity, a large gill surface area, and the extension of the lower lip facilitate this phenomenon. An increase of the hemoglobin content and the erythrocytes were also observed. However, no data were provided for larval *P. mesopotamicus* besides the conclusion that at this stage fish were able to survive for 10 min or 3–5 h without or with access to water surface, respectively. The results of the present study indicated that under low dissolved oxygen concentration (2.2-mg O_2/L), reproductive performances of *P. brachypomus* were significantly altered, including changes in steroid hormone concentrations, ovulation, and egg fertilizing ability. Viability of the larvae was also affected. Although we were not able to separate the effect of oxygen concentrations on reproductive efficiency and embryonic development, it was evident that concentrations as low as 4.0 mg/L (semi-normoxic conditions) were tolerated during embryonic development of this species and did not result in larval deformities. As demonstrated by Rombough (1988) in rainbow trout, critical oxygen concentrations increased gradually during embryonic development (1–8 mg O_2/L) and depended on water temperature. Further studies should address specific periods of embryogenesis of tropical fish where hypoxia results in embryos deformities and mortality.

Under normoxic conditions, conceptual (LHRHa) was used successfully to induce spermiation and final maturation/ovulation in *P. brachypomus.* The embryo survival (68.5 ± 25%) was comparable to tambaqui *Colossoma macropomum,* a closely related species, in which ovulation was induced using carp pituitary extracts (CPE) or human chorionic gonadotropin (HCG) (Chellappa et al. 1996). In contrast, under hypoxia the effectiveness of the hormonal treatments was reduced.

The changes of plasma sex steroids during final maturation/ovulation in *P. brachypomus* were similar to those reported in salmonids. The increase of plasma levels of T associated with the drop of E2 observed at the time of ovulation reflected the decrease of the aromatase activity in the ovary (Fostier et al. 1983). As reported by Gazola et al. (1996) in another characid fish *P. mesopotamicus,* a surge of 17,20βP was observed only at the time of ovulation. This steroid has been shown to be one of the most potent steroids for inducing final oocyte maturation and was found at high concentrations in the plasma of ovulating females of teleost fishes (Nagahama 1987; Nagahama and Yamoshita 1989). Therefore, it appears that 17,20βP is likely the maturational inducing steroid (MIS) in Serrasalmidae. However, we cannot exclude the possibility that other steroids, such as 17,20β,21-trihydroxy-4-pregnen-3-one (20βS) might be involved in the maturational process of the oocytes. Further studies are required to confirm that 17,20βP is the MIS in Serrasalmidae.

Although the patterns of the plasma sex steroids in females were similar regardless of the oxygen concentration, their levels under hypoxic conditions were depressed in comparison to normoxia. Environmental changes such as decrease of dissolved oxygen are considered to induce a stress response in fish. Acute and chronic stresses induced a decrease of plasma androgens and estrogens in most species (Sumpter et al. 1987; Carragher and Pankhurst 1991; Pankhurst and Van der Kraak 1997) and consequently affected the quality of the eggs and the development of the fish larvae. Most importantly, in salmon exposed to

confinement stress, which involved disturbance in oxygen supply, the level of 17,20βP decreased significantly after only 30 min (Kubokawa et al. 1999). This fact can be linked to delayed ovulation. In the present study, the low levels of E2 and T reported prior to hormonal injections in females maintained under hypoxia did not affect egg size. However, final maturation/ovulation was not achieved in all females, embryo survival was significantly lower (17.3 ± 28%), and a high percentage of embryos with deformities was observed (91 ± 6%). Body deformities are commonly observed in teleost embryos incubated in hypoxic conditions (Keckeis et al. 1996). Campbell et al. (1992, 1994) also observed lower survival of progeny of rainbow and brown trout stressed during late vitellogenesis. In Atlantic cod, a higher incidence of deformed larvae was reported when parents were subjected to netting stress 3 times a week, although egg production fertilization and survival between progeny of stressed and unstressed fish were similar (Wilson et al. 1995). Maternally derived cortisol and T are important in regulating growth, development, and nutrient reserves of the embryo and larvae (McCormick 1999). We did not measure cortisol and T levels in the eggs. However, the concentrations of T that were significantly lower in females maintained under hypoxia may have consequently impacted larval survival.

In contrast to females, all males responded to the hormonal treatments. Spermiation was associated with a significant increase of plasma T and 17,20βP and a decrease of the concentration of 11-kT. The levels measured in the present study were higher than those reported by Gazola and Borella (1997) in *P. mesopotamicus*. As in females, the levels of plasma androgens were significantly different between fish under normoxia and hypoxia. Low plasma concentrations of T and 11-kT were also reported in male brown trout exposed to acute handling and confinement stress for 1 h (Pickering et al. 1987). Sperm characteristics, including sperm concentration, LDH, and protein concentration in seminal plasma and osmolality, were not altered by exposure to low oxygen concentrations.

In the present study, we provide evidence that oxygen concentrations have significant effects on final maturation/ovulation in tropical fish *P. brachypomus*. We also observed that under hypoxic conditions, survival of embryos was severely impacted. Therefore, we recommend a concentration of oxygen of at least 4 mg/L to ensure adequate reproductive performances and embryonic development of pacu.

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