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Temperature induces gonadal maturation and affects electrophysiological sexual maturity indicators in *Brachyhypopomus pinnicaudatus* from a temperate climate

Laura Quintana[1,*], Ana Silva[1,2], Nibia Berois[3] and Omar Macadar[1]

[1]*Depto de Neurofisiología, Instituto de Investigaciones Biológicas Clemente Estable, Unidad Asociada de Facultad de Ciencias – Universidad de la República, Avda, Italia 3318, Montevideo, Uruguay*, [2]*Depto de Fisiología* and [3]*Sección de Biología Celular, Facultad de Ciencias- Universidad de la República, Montevideo, Uruguay*

*Author for correspondence (e-mail: lauraq@iibce.edu.uy)

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Summary

In contrast to most of the previous studies in gymnotiform reproduction, which have been conducted in the tropical region, this study examines a gymnotid from the temperate region in both the natural habitat and the laboratory. The gonadal histology of *Brachyhypopomus pinnicaudatus* is described for the first time. The male had a paired, lobular testis of the unrestricted spermatogonial type, and females a paired saccular cystovary. Analysis of gonads and their annual cycle enabled us to confirm the breeding season and to conclude that this species is a multiple spawner. Water temperature and photoperiod showed the expected annual cycles for the region. High temperature and a 14 h:10 h L:D photoperiod in the natural habitat coincided with (1) mature gonadal stages, (2) electrophysiological sexual dimorphism: males present a lengthened negative phase in their electric organ discharge (EOD) and (3) decreased temperature sensitivity of the EOD: the waveform does not change when temperature increases above 20°C. Acclimation to sustained high temperature (30 days, 28°C, 12 h:12 h L:D, low conductivity) induced gonad maturation along with EOD dimorphism. Our data show that high environmental temperature is enough to trigger sexual maturity in *Brachyhypopomus pinnicaudatus* from a temperate climate.

Key words: breeding, temperature sensitivity, electric fish, *Brachyhypopomus pinnicaudatus*, EOD.

Introduction

Fishes of the order Gymnotiformes (Mago-Leccia, 1994) possess electric organs (EOs) that generate electric discharges (EODs) with species-specific waveforms. In addition, they have electroreceptors specifically tuned to detect currents generated by their own EOs. This sensory modality (active electroreception) allows the fish to perceive objects with electrical properties different from the surrounding water (electrolocation; Lissmann, 1958; Bastian, 1986) and to communicate with conspecifics (electrocommunication; Hopkins, 1972, 1986).

Communication becomes essential during the breeding season, when fish need to identify sexually mature, receptive partners of the same species. Reliable and detectable modulations in EODs could serve as signals of reproductive state (Kelly, 1983). In *Brachyhypopomus pinnicaudatus* the EOD is biphasic (in a head-to-tail recording), composed of an early head-positive wave (P1) and a late head-negative wave (P2) (Hopkins, 1991). During the breeding season, in mature males, the EOD increases its duration *via* a longer P2 phase (Hopkins et al., 1990; Hopkins, 1991; Caputi et al., 1998; Silva et al., 1999). This reversible effect upon the EOD has been shown in a related species, *Hypopomus occidentalis*, to depend on androgen levels acting directly on the EO (Hagedorn and Carr, 1985). *B. pinnicaudatus* also exhibits morphological dimorphism during the breeding season (Hopkins et al., 1990; Hopkins, 1991; Caputi et al., 1998; Silva et al., 1999, 2003). In mature males the tail filament becomes noticeably wider whereas females display protruding ovaries through their translucent skin.

The geographical distribution of Gymnotiformes is broad: from the Chiapas Province in Mexico in the north (Vilano and Balderas, 1987) to the Río de la Plata system in the south (Mago-Leccia, 1994). Over this large geographical area, different environmental cues, which change seasonally and trigger the onset of breeding in fishes – henceforth referred to as 'zeitgebers' – have been identified (Hopkins, 1974a,b; Schwassmann, 1976; Provenzano, 1984; Hagedorn, 1988; Silva et al., 1999, 2002, 2003). Previous studies have been conducted in the tropical region where, as with many other Neotropical fishes, most Gymnotiformes breed during the rainy season (Hopkins, 1974a,b; Kirschbaum, 1979; 1995b). Nevertheless, differences in spawning cues have been revealed even within the same species (Schwassmann, 1976; Hagedorn, 1986, 1988).

Reports on gonadal histology of Gymnotiformes, as well as its annual changes, are scarce. Moreover, the few existing studies have been done in Gymnotiformes from the tropical zone. Features of germ cells were briefly described for *Gymnotus carapo* (Barbieri and Barbieri 1984, 1985) and *Stenopygus macrurus* (Zakon et al., 1991). In Gymnotiformes of the tropical region, gonadal cycles have been temporally correlated to the alternation of the rainy and dry seasons (Provenzano, 1984; Zakon et al., 1991).

In temperate and subtropical regions, photoperiod, temperature and their interaction are demonstrated zeitgebers of reproductive cycles (Lam, 1983; Prosser and Heath, 1991). In Uruguay (30–35°S), the southern boundary of gymnotiform distribution in America, both water temperature and photoperiod change throughout the year, whereas rainfall does not show seasonal changes (Silva et al., 1999, 2002, 2003). We selected temperature and have analyzed it as the main zeitgeber in the reproduction of *B. pinnicaudatus* at this latitude (Silva et al., 1999, 2002, 2003).

Temperature variations within the natural range for the temperate climate have a short-term effect upon EOD waveform in *B. pinnicaudatus* (Caputi et al., 1998; Silva et al., 1999, 2002). The duration of the *B. pinnicaudatus* EOD diminishes as temperature increases. In addition there is a striking decrease in the amplitude of P2 in water at temperatures higher than 20°C (which reverses once the temperature is returned to control values); this effect has been called ‘waveform temperature sensitivity’ (Caputi et al., 1998; Silva et al., 1999, 2002). This effect upon EOD waveform is so important that at 30°C the EOD is almost monophasic. We observed this high temperature sensitivity in juveniles and in non-differentiated adult fish (fish that do not present sexual dimorphism), whereas fish collected in the breeding season displayed very low temperature sensitivity (in water at 30°C, changes in amplitude were transient and returned to control values in approximately 10 min) (Caputi et al., 1998; Silva et al., 1999, 2002). Similar observations of this phenomenon in *Gymnotus carapo* have been reported (Ardanaz et al., 2001) and were used to prove its peripheral origin.

The aims of this study were: (i) to analyze the histological features of gonads and their annual changes in wild *B. pinnicaudatus* in relation to water temperature and conductivity; (ii) to analyze annual changes of electrophysiological features linked to the occurrence of the breeding season (EOD waveform and temperature sensitivity), (iii) to evaluate the effect of high temperature acclimation upon gonad histology and EOD waveform.

Materials and methods

Collections and experiments were done under the guidelines of the Ministerio de Ganadería, Agricultura y Pesca, Uruguay, and the Society for Neuroscience. Our recording conditions did not include traumatic maneuvers and fish did not show signs of pain (as judged by escape responses or sudden changes in the EOD rate).

Sampling

Fish *Brachyhypopomus pinnicaudatus* (Hopkins) were captured by taking advantage of gymnotiform electrogeneration capability and of their nocturnal habits. They were collected during daytime, when at rest in their hiding places, using a ‘fish detector’ (a portable electrode amplifier loudspeaker assembly that detects the presence of fish from a distance up to 2 m) and a hand net. The fish were collected from a small lake, El Tigre, (Department of Treinta y Tres, 33°18′S, 54°35′W) and neighboring sites. During 1999 and 2000, samples were taken once a month (spring–summer) or every 2 months (autumn–winter). A total of 89 individuals were used in this study. Temperature and conductivity measurements were taken at midday (digital thermometer TM-915; Lutron, Montevideo, Uruguay; TDSu Testr 3; Cole Parmer, Chicago, USA, respectively). *B. pinnicaudatus* were identified using morphological and electrophysiological cues (Hopkins, 1991). Adults measured 12–18 cm. Adults collected in the non-breeding season did not present any characteristics of sexual dimorphism, and are referred to as ‘non-differentiated fish’.

Histology

Animals were subjected to hypothermia and killed by decapitation. Gonads were removed immediately afterwards and were fixed in Bouin’s solution, dehydrated in ethanol and embedded in paraffin wax. Sections (5–7 μm thick) were stained with Hematoxylin and Eosin and mounted in Entellan (Merck, Darmstadt, Germany) (Ganter and Jolles, 1970). Sections were examined and photographed under an Olympus–Vanox light microscope (Kodak Gold, 100 ASA, 35 mm film). Measurements of germ cell size were carried out directly under the microscope using an ocular micrometer (1/100 mm; E. Leitz, Wetzlar, Germany). An average of 35 cells per stage were measured. To evaluate if the different cell types were present throughout the entire gonad, 3 ovaries and 4 testes of fish captured in October were entirely sectioned from rostral to caudal end. In both cases it was clear that cell types were not polarized and were present along the entire axis. We therefore worked with sections of the mid gonad. A total of 70 individuals (40 males and 30 females) were used in this analysis.

To quantitatively compare the distribution of different cell types throughout the annual cycle, three months were selected: July, October (non-breeding season) and December (breeding season). We analyzed three different sections of each fish, and four fish were used in each group. Germ cells were observed and classified according to their sizes and main morphological characteristics. Quantification of male germ cells was done using a Whipple–Hauser grid with a total of a hundred squares. Each square was counted as having the cell of the predominating cyst, and the results were expressed as percentage of total cells of the section. Quantification of female germ cells was done by measuring the area each cell type covered, and the results were expressed as percentage of total area of the section (relative area). All measurements were done using computer software (Image Tool, San Antonio, USA).

EOD recordings

Head-to-tail EOD recordings were obtained as described by Caputi et al. (1998) and Silva et al. (1999) throughout the experiments. Fish were placed in a perforated plastic cylinder (3 cm diameter, length adapted to the fish's length) with two Nichrome electrodes (at opposite ends) connected to a high input impedance (GΩ) buffer amplifier. The cylinder was immersed in a 30 cm×20 cm×15 cm tank. The outputs of the amplifier were connected to a digital oscilloscope. Averages of 64 EODs were stored in a PC for further analysis with software developed in the laboratory. This recording device restricted fish movements that could interfere with EOD recordings. Water temperature was changed following different protocols (see below) and recorded from inside the cylinder. In order to favor comparisons, EODs were normalized in amplitude with respect to P1. No voltage calibration will therefore be included. P1 and P2 durations were measured at 10% of peak amplitude. The lengthening of P2 duration typical of the mature breeding male was chosen as an indicator of electrophysiological dimorphism. In order to avoid the influence of temperature on EOD duration, we quantified electrophysiological dimorphism by the duration of P2/duration of P1 (DP2/DP1) ratio.

Temperature effects on EOD waveform

The experiments carried out to evaluate the temperature sensitivity of EOD waveform are detailed elsewhere (Caputi et al., 1998; Silva et al., 1999; Ardanaz et al., 2001). Briefly, fish were placed in 20°C water for 1 h, after which their EOD was recorded and kept as control value. The recording cylinder was then gently lifted, drained and submerged in water at 30°C. EOD recordings were obtained 30 min after the temperature step was imposed. The peak amplitude of P2/peak amplitude of P1 (AP2/AP1) ratio was used to represent waveform changes with temperature. A temperature sensitivity waveform index (TS index) was constructed with the AP2/AP1 measured at 20°C (control) and 30°C (after 30 min), the difference being divided by the value at 20°C, that is:

$$\text{TS index} = [(\text{AP2/AP1})_{20°\text{C}} - (\text{AP2/AP1})_{30°\text{C}}] / (\text{AP2/AP1})_{20°\text{C}} .$$

This relationship was used to evaluate temperature sensitivity when fish were submitted to temperature changes.

Acclimation experiments

Non-differentiated adults were acclimated at a constant temperature of 28°C for 30 days with a 12 h:12 h L:D photoperiod and constant conductivity (~100 μS cm^{-1}) following the same procedures as described by Silva et al. (1999). Briefly, conductivity was controlled daily and kept low and constant by addition of distilled water; fish shared tanks in pairs and were fed every other day. To evaluate changes in gonadal profile, fish collected in July were divided in two groups: a control group, which was recorded before histological examination (*N*=7: 3 males and 4 females), and a group that was acclimated during the month of August (*N*=6: 4 males and 2 females); EOD recordings were then performed before gonad histological examination. The same protocol was repeated with a group of fish collected in September (control group *N*=6: 4 males and 2 females; acclimated group *N*=6: 4 males and 2 females).

Results

Female gonad histology in B. pinnicaudatus

Females presented two ovaries that ran ventrally along the abdominal cavity; the two lobes extended posteriorly and were attached to the dorsal wall of the cavity. Anteriorly, the two ovaries united to form a common structure, here referred to as the common genital sinus. This extended anteriorly to a genital papilla, which exited ventrally between the opercula, together with the anus.

The ovigerous lamellae, covered by germinal epithelium, extended from the ovary wall to the center of the organ. Oocytes grew as a cluster within each lamella. Five different germ cell types were recognized inside the lamellae (Table 1). Oogonias were round cells that had scarce cytoplasm, a central nucleus and a single nucleolus. They were observed alone or in small aggregations (Fig. 1A). Previtellogenic oocytes were round or polygonal and presented strongly basophilic cytoplasm. They had a central, spherical nucleus, with chromatin observed in fine granules (Fig. 1B). In larger previtellogenic oocytes, numerous nucleoli close to the nuclear membrane could be seen, as well as lampbrush chromosomes. Oocytes in the lipid yolk stage were present in a wide range of sizes. Lipid droplets started to appear peripherally and, as the oocytes grew, the droplets increased in number and size and invaded the cytoplasm inwardly. The nucleus contained many nucleoli and sometimes appeared deformed by the large surrounding lipid droplets. At this stage the chorion (acellular membrane located between follicle cells and oocyte) started to be visible. In these oocytes the width of the chorion was 5.21±3.39 μm (mean ± S.D.; Fig. 1C). Acidophilic yolk (or protein yolk) oocytes presented yolk granules that started to appear close to the nucleus. The chorion was 8.87±2.40 μm in width and did not differ significantly from chorions of advanced protein yolk oocytes. In advanced stages, the protein yolk had pushed the lipid yolk to the periphery and the chorion was 7.38±2.28 μm (Fig. 1E). In both lipid and protein yolk oocytes, radial stratification could be observed in the chorion. Fully grown oocytes contained a very large amount of protein yolk which paraffin wax did not penetrate entirely. This methodological disadvantage made it very difficult to observe intact fully grown yolk oocytes and did not allow sections of preovulatory oocytes. However, morphological features from remaining parts of fully grown oocytes allowed their identification. Both atretic follicles and post-ovulatory follicles were also observed.

Male gonad histology in B. pinnicaudatus

The testis is a paired organ, of a more-or-less triangular section, positioned ventrally and extending posteriorly to the dorsal wall of the abdominal cavity. The gonads joined

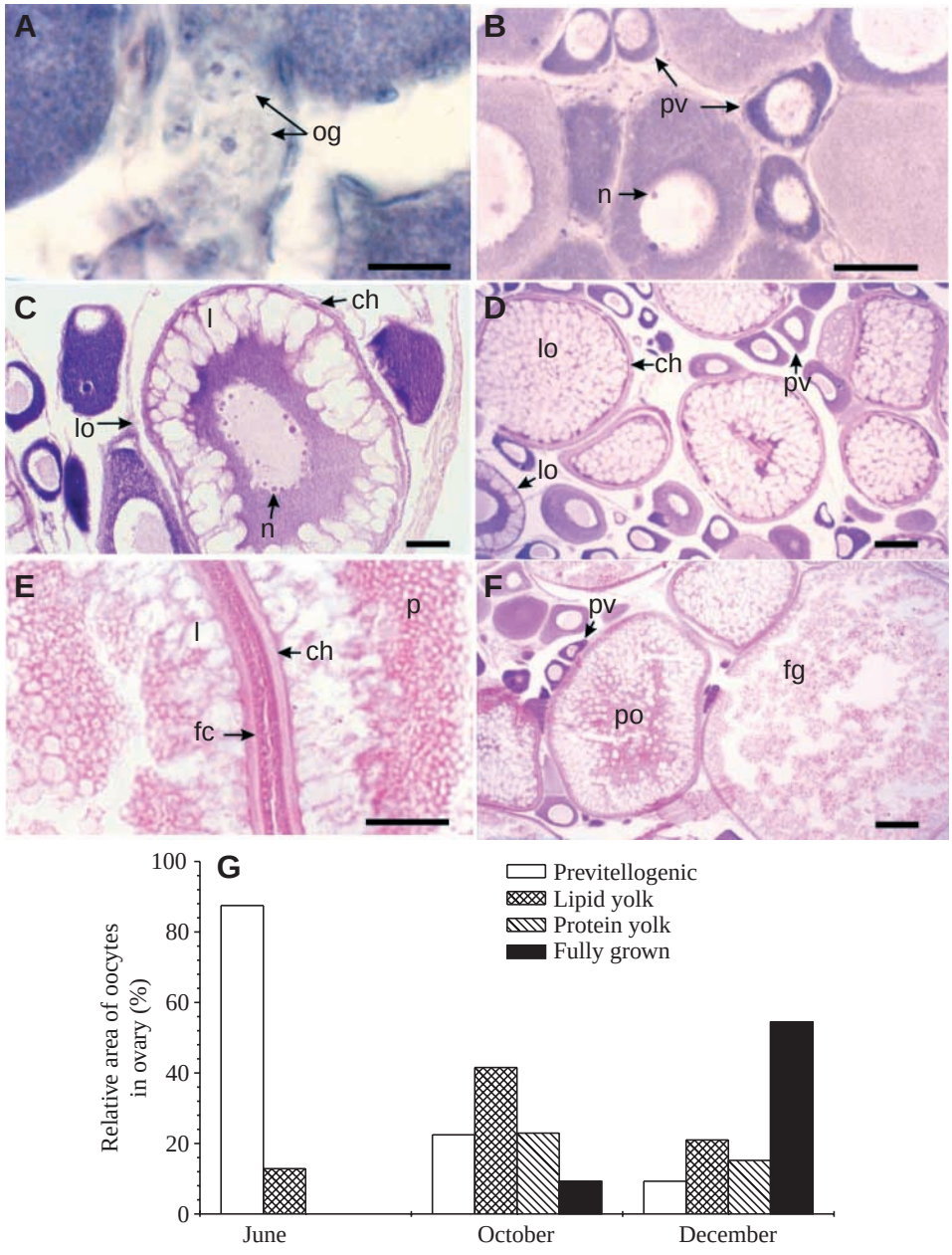

Fig. 1. Germ cell features (A,C,E) and panoramic views of ovaries (B,D,F). (A) Small aggregation of oogonia. Note the large, pale nucleus, single nucleoli and scarce cytoplasm. Scale bar, 10 μm. (B) Ovary in the resting stage from a fish captured in July. Previtellogenic oocytes show a very basophilic cytoplasm and various perinuclear nucleoli. It is possible to observe oocytes in different sizes, some may be in very early stages of vitellogenesis. Scale bar, 50 μm. (C) Oocyte in lipid yolk stage. Note the lipid droplets, the numerous perinuclear nucleoli and the chorion. Scale bar, 50 μm. (D) Ovary in the recovering stage from a fish captured in October. Oocytes are mainly previtellogenic and lipid yolk. Scale bar, 100 μm. (E) Two oocytes in the protein yolk stage. The protein yolk has pushed the lipid droplets to the periphery. The chorions are wide. Note the follicular cells between the oocytes. Scale bar, 50 μm. (F) Mature ovary from a fish captured in December. It is possible to observe oocytes in all stages of development. Fully grown oocytes, others in process of protein yolk deposition, lipid yolk oocytes and previtellogenic oocytes. Scale bar, 100 μm.. The maturing ovary, present in October and November is not shown. (G) Cell-type distribution represented by relative area of four different types of oocytes in ovaries of freshly captured females in June (*N*=4, non-breeding season), October (*N*=4), and December (*N*=4, breeding season). White, previtellogenic oocytes; cross-hatched pattern, lipid yolk oocytes; hatched pattern, protein yolk oocytes; black, fully grown oocytes. Previtellogenic oocytes are present throughout the year. ch, chorion; fg, fully grown oocyte; fc, follicular cells; l, lipid yolk; lo, lipid yolk oocyte; n, nucleolus; og, oogonia; p, protein yolk; po, protein yolk oocyte; pv, previtellogenic oocyte.

anteriorly in a common genital sinus, which connected to a genital papilla located ventrally between the opercula. Testicles were organized in a lobular testicular structure consisting of branching tubuli and interstitial tissue among them. The seminiferous tubule had a central lumen surrounded by the germinal epithelium. This was organized in cysts, where spermatogenesis occured. Sertoli cells formed the wall of the cyst. Each cyst contained germ cells in the same stage of development. Four different cell types were identified inside the cysts (Table 2). Spermatogonia were large cells with a very conspicuous and spherical nucleus. It was possible to see the peripherical condensed chromatin, whereas the cytoplasm was very scarce and lightly basophilic (Fig. 2A). Spermatocytes were smaller in size and their nucleus contained granular chromatin in different stages of condensation depending on the development of the spermatocyte (Fig. 2C). Spermatids, which were in the cysts, were small and the nucleus was strongly basophilic (Fig. 2C). Late spermatids were observed ‘oriented’ inside the cysts, with their heads towards the periphery and their tails towards the center (Fig. 2E). Finally, free sperm in the lumen were smaller still and showed a strongly basophilic nucleus (Fig. 2E).

Annual cycle of gonads

Four histological phases were used to describe the ovarian annual cycle: regressing/resting, recovering, maturing and

Table 1. Brachyhypopomus pinnicaudatus *oogenesis*

Stage	Diameter (μm)	Nucleus	Nucleoli	Cytoplasm	Chorion
Oogonia	5–13	Spherical, central Fibrillar chromatin	1	Scarce	–
Previtellogenic oocyte	20–175	Spherical, central Fibrillar chromatin Lampbrush chromosomes	Several Close to nuclear membrane	Strongly basophilic	–
Lipid yolk oocyte	165–550	Usually deformed, central Fine chromatin	Several, small	Basophilic Foamy lipid droplets	Starts deposition
Protein yolk oocyte	500–850	Usually deformed, central Fine chromatin	Several, small	Peripheric lipid yolk Perinucleus protein yolk	Continues deposition
Fully grown oocyte	800–1250	Usually deformed, central Fine chromatin	Several, small	Abundant protein yolk Scarce and peripheric lipid yolk	Continues and completes deposition

Female germ cells were classified according to their size and main morphological characteristics.

Table 2. Brachyhypopomus pinnicaudatus *spermatogenesis*

Stage	Diameter (μm)	Nucleus	Cytoplasm	Location
Spermatogonia	5–10	Spherical Peripheric condensed chromatin	Lightly basophilic	Isolated or in small groups
Spermatocyte	3–5	Spherical, granular chromatin	Thin	Grouped in cysts
Spermatid	1.5–2.5	Spherical, strongly basophilic	Scarce	Grouped in cysts
Spermatozoa	0.5–2	Spherical, strongly basophilic	Practically non visible	Mainly free in lumen

Male germ cells were classified according to their size and main morphological characteristics.

mature. At all stages of the cycle previtellogenic oocytes were present. The regressing/resting ovary was present from February to July; this stage indicated the end of the spawning season of the species. It was characterized by hemorrhagic connective tissue, oogonia and small previtellogenic oocytes. The ovary gradually started to increase the number of previtellogenic oocytes, and in July some ovaries started the recovering stage (Fig. 1B). The recovering ovary was observed between August and October; most oocytes were in the process of growth and deposition of lipid yolk (Fig. 1D). They were in lamellae with well-defined limits. The maturing ovary was present in October and November. It contained mainly two kinds of oocytes: with lipid yolk or protein yolk, the latter in different states of deposition and growth. The lamellae lacked well-defined limits due to the size of the oocytes. The mature ovary was found from November to January. It consisted mainly of large fully grown oocytes while a few others were still in the process of deposition of protein yolk (Fig. 1F). In addition, atretic follicles could be observed as well as post-ovulatory follicles, which indicated spawning. The relative area covered by oocytes showing different degrees of maturation, varied throughout the year as can be observed in Fig. 1G (N=4 for each experimental group).

Four histological phases were used to describe the testicular annual cycle: regressing/resting, recovering, maturing and mature. The regressing/resting testicle was observed from February to July. At the beginning of this period hemorrhagic tissue was present and the germ cells were mostly spermatogonia with very few pools of unspawned sperm. Towards June and July testis showed an almost homogeneous population of large spermatogonia and an occasional cyst of spermatocytes (Fig. 2B). The recovering testis was observed from August to October. It was possible to observe some spermatogonia and cysts of spermatocytes in different stages of development, as well as spermatids (Fig. 2D). The maturing testis was found in October and November. It contained mainly cysts of spermatocytes and spermatids, many of which were oriented inside the cysts. The mature testis was observed from December to February. Spermatids in different stages of development were observed as well as large pools of sperm in the lumen. The germinal epithelium had become thinner (Fig. 2F). The gonadal profile, represented in Fig. 2G as the

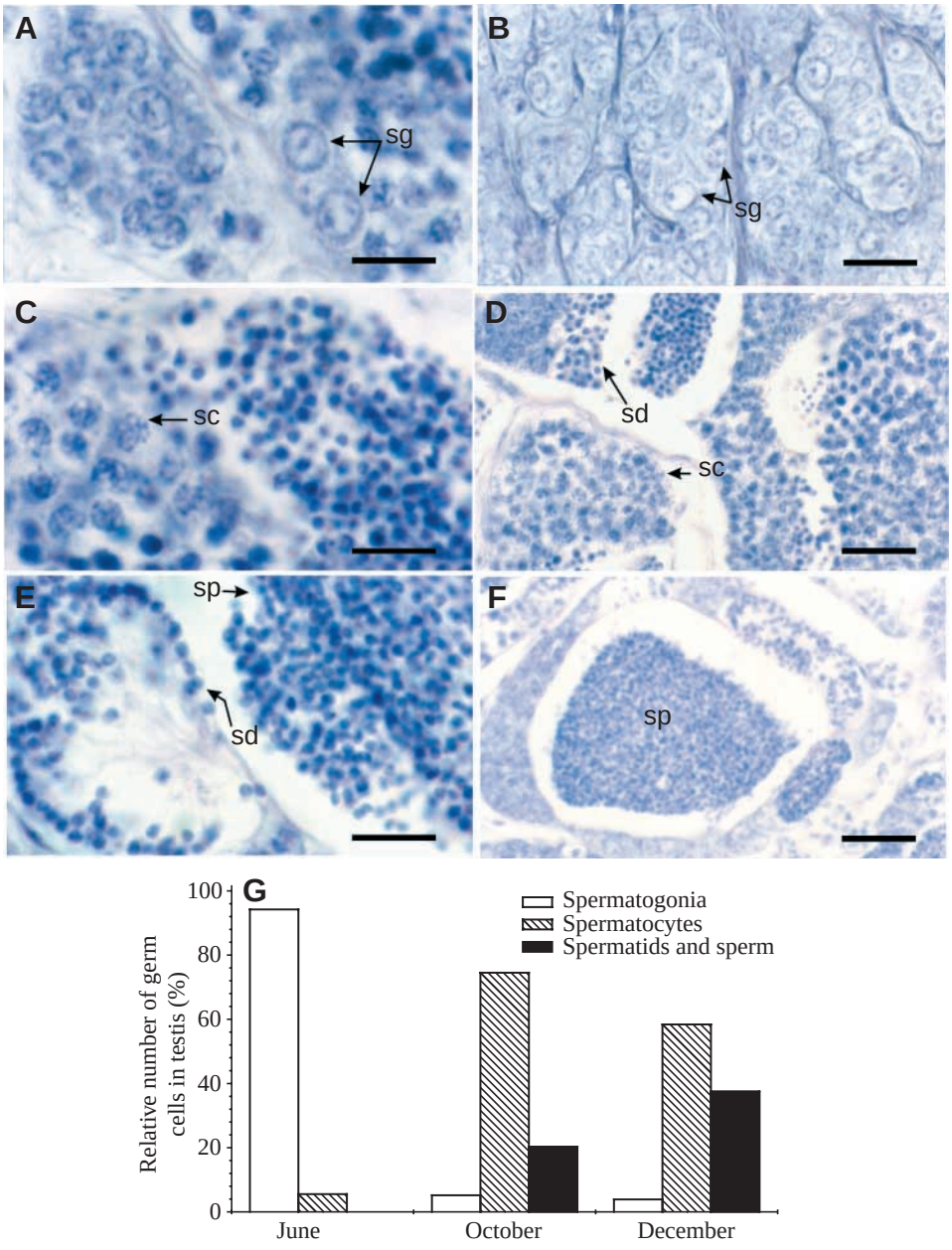

Fig. 2. Germ cell features (A,C,E) and panoramic views of testes (B,D,F). (A) Two very early spermatogonia which have peripherical condensed chromatin in their nucleus, a single nucleolus and lightly colored and scarce cytoplasm. On the left hand side of the picture it is possible to observe a cluster of spermatogonias that have probably undergone further mitosis, given their smaller size. Scale bar, 10 µm. (B) Resting testis from a fish captured in July. The tubules are filled with spermatogonia. Scale bar, 20 µm. (C) Cyst of early spermatocytes. Note that the chromatin is in the initial phase of condensation. Scale bar, 10 µm. (D) Recovering testis from a fish captured in October. The tubules are mainly filled with cysts of spermatocytes. It is also possible to observe cysts of spermatids. Scale bar, 20 µm. (E) Spermatids oriented inside the cysts, with their heads towards the periphery and their tails towards the center. On the right hand side there is free sperm in the lumen Scale bar, 10 µm. (F) Mature testis from a fish captured in December. The germinal epithelium has become thinner, cysts of spermatocytes remain but testis is dominated by pools of free sperm in the lumen. Scale bar, 20 µm. The maturing testis, present in October and November is not shown. (G) Relative distribution of three different groups of germ cells in testis of freshly captured males in June (*N*=4, non-breeding season), October (*N*=4), and December (*N*=4, breeding season). White, spermatogonia; hatched pattern, spermatocytes; black, spermatids and sperm. sc, spermatocyte; sd, spermatid; sg, spermatogonia; sp, sperm.

relative number of different germ cells throughout the year, showed the expected annual cycle (*N*=4 for each experimental group).

Annual cycle of environmental variables

The breeding season of *B. pinnicaudatus* has been reported to occur from November to January (Silva et al., 2003). We studied the annual cycle of environmental variables in one same habitat over 2 years. Water conductivity was low and relatively constant throughout the study period (47±21.62 µS cm^{-1} in 1999 and 53.75±40.68 µS cm^{-1} in 2000: Fig. 3A). Rainfall occurs all year long (raw data obtained from National Weather Service, Uruguay. Seasonal data was obtained over the last 30 years; summer: 312±84 mm; autumn: 302±79 mm; winter: 263±89 mm; spring: 307±84 mm) and does not show significant differences between seasons (ANOVA, *P*=0.188). Water temperature measurements, always taken at noon, showed the expected seasonal changes, ranging from 11°C in June to 34°C in January and February, as shown in Fig. 3C.

Annual changes of electrophysiological characteristics

Electrophysiological dimorphism of the male, measured by the ratio DP2/DP1, was approximately 1 in the non-breeding season and gradually increased towards the summer, reaching its peak mean value, 1.52, in December (Fig. 3D). By contrast, the temperature sensitivity of the EOD waveform increased towards the winter, and decreased towards the summer (Fig. 3E). Its lowest mean value, 0.66, coincided with high water temperatures, marked male electric dimorphism and

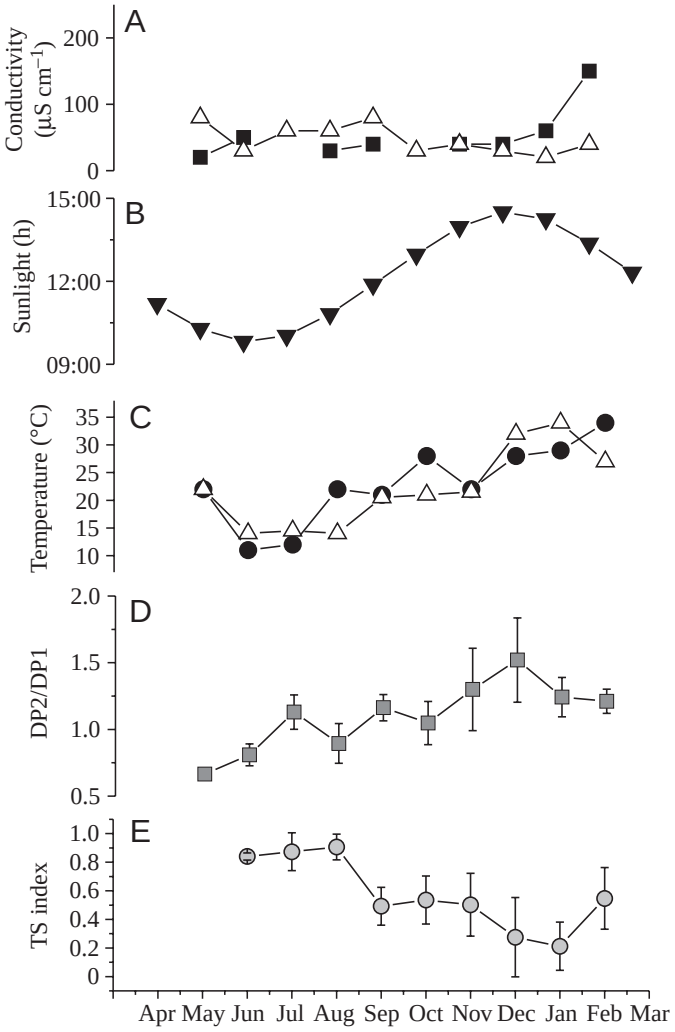

Fig. 3. Annual cycle of physicochemical water parameters and electrical characteristics of *Brachyhypopomus pinnicaudatus* in the wild. (A) water conductivity, (B) sunlight, (C) water temperature, (D) male electric dimorphism (DP2/DP1), (E) EOD temperature sensitivity index (TS). In A and C, solid and open symbols represent values taken during 1999 and 2000, respectively.

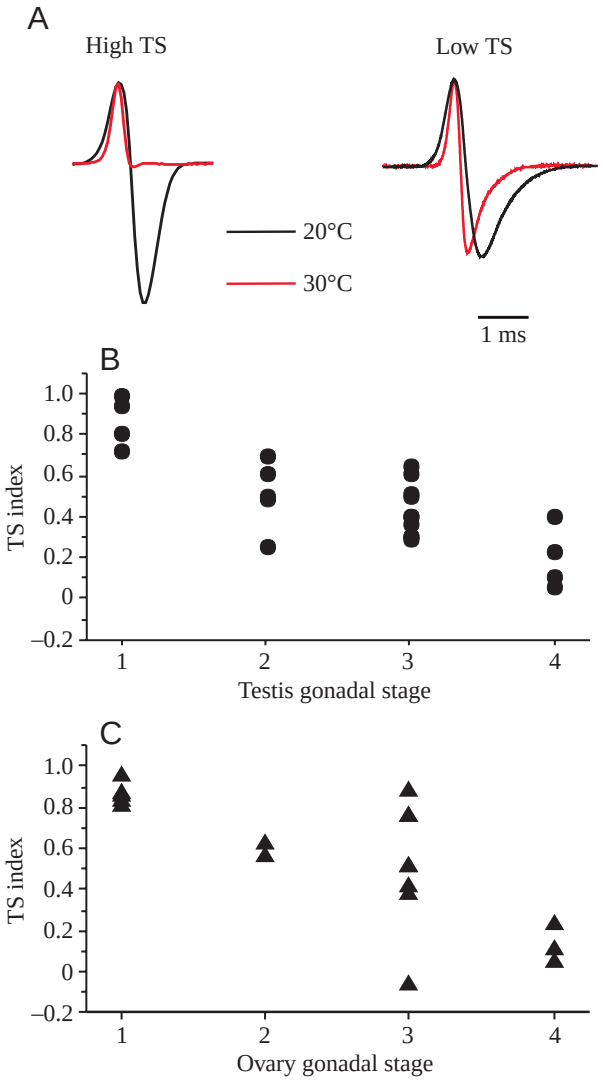

Fig. 4. Temperature sensitivity of EOD waveform. (A) Recordings of EOD waveform at 20°C (black) and after 30 min at 30°C (red) in males with high (left) and low (right) temperature sensitivity. Fish with high temperature sensitivity lose their negative component (P2) at 30°C whereas the amplitude of the negative component is not different at 20° and 30°C in fish with low temperature sensitivity. (B,C) Temperature sensitivity is inversely correlated to gonadal stages of maturity. Stage 1, regressing/resting; stage 2, recovering; stage 3, maturing; stage 4, mature. TS index is high in males and females that present resting gonads and low in fish with mature gonads (Mann–Whitney test, $P<0.05$; $n_1=4$, $n_2=4$).

extreme photoperiod (14 h:10 h L:D). It is clear that gonads reached stages of maturity in coincidence with high water temperature and extreme photoperiod. Moreover, gonadal maturity appeared together with electrophysiological dimorphism and a low TS index.

EOD recordings of two fish, a non-differentiated adult and a mature male, subjected to a temperature step from 20° to 30°C are shown in Fig. 4A. At 20°C both fish showed the typical biphasic EOD, whereas after 30 min at 30°C the non-differentiated adult presented a practically monophasic EOD. This fish presented high temperature sensitivity (Fig. 4A, left). However, at 30°C, the mature male presented an EOD similar in amplitude to the control recording, thus displaying low temperature sensitivity (Fig. 4A, right). TS indices of fish with resting/regressing gonads (stage 1 in Fig. 4B,C) were significantly higher than the values presented by fish with mature gonads (stage 4 in Fig. 4B,C) in both males and females (Mann–Whitney test, $P<0.05$; $n_1=4$, $n_2=4$).

Induction of sexual maturity by acclimation

To evaluate the role that temperature may play in the onset of the breeding season, acclimation experiments were carried out (28°C, constant and low water conductivity, 12 h:12 h L:D). The effects of acclimation upon electrophysiological dimorphism and gonad histology were analyzed.

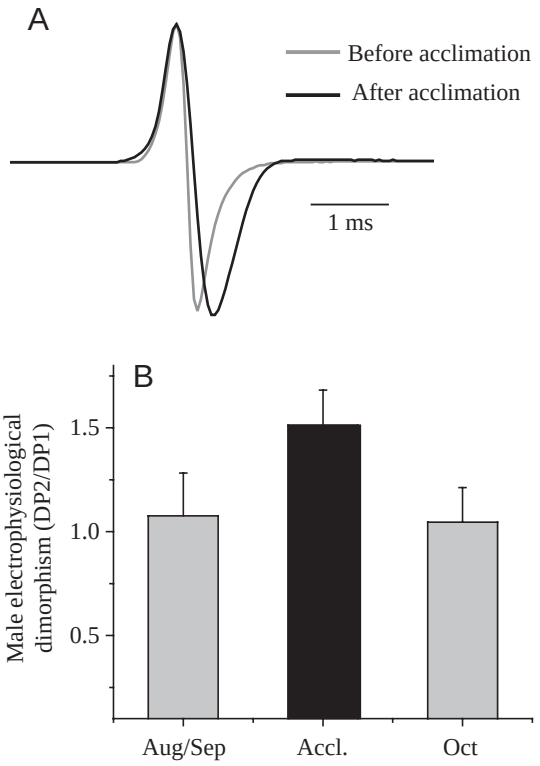

Fig. 5. Acclimation induces electrophysiological dimorphism in males of *Brachyhypopomus pinnicaudatus*. (A) Normalized EOD recordings of a male captured in July before (grey line) and after (black line) acclimation (30 days at 28°C, constant photoperiod and water conductivity). (B) Electrophysiological dimorphism of the male, measured by the duration of P2/duration P1 (DP2/DP1) ratio, of males captured from the natural habitat in August/September and October (grey bars) and males captured in August/September and acclimated for 30 days at 28°C (black bar). The DP2/DP1 ratio was significantly higher after acclimation than those obtained in freshly captured fish (Mann–Whitney test, $P<0.05$).

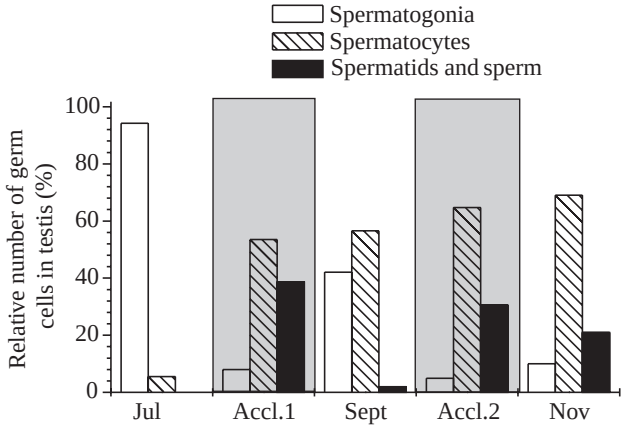

Fig. 6. Acclimation induces gonadal maturity in males. The relative number of spermatogonia, spermatocytes and spermatids/sperm present in the testis were assessed in five experimental groups. Jul, fish captured in July; Accl.1, fish captured in July and acclimated for 30 days at 28°C (constant photoperiod and water conductivity); Sept, fish captured in September; Accl.2, Fish captured in September and acclimated for 30 days at 28°C (constant photoperiod and water conductivity); Nov, fish captured in November. Testes of males freshly captured in July have spermatogonia and spermatocytes. After acclimation (grey) the cell profile changed and resembled a mature male, presenting almost 40% of advanced germ cells (spermatids and sperm). The experiment was repeated with a group of fish collected in September, with similar results. White bars, spermatogonia; hatched bars, spermatocytes; black bars, spermatids and sperm.

Non-differentiated males were acclimated to high temperature for 30 days. In contrast to the effects of sustained low-temperature acclimation, which induces a decline in morphological and electrophysiological signs of sexual maturity (Silva et al., 1999; see Discussion), the EOD waveform after 28°C-acclimation showed a lengthened P2 phase in comparison to the waveform before the experiment (Fig. 5A). Males captured in September and acclimated during 30 days at 28°C were compared to those of the natural habitat captured before the acclimation experiment (August/September). A second control group was captured from the natural habitat after the acclimation experiment was over (October). These two control groups did not present significant differences in the DP2/DP1 ratio (Figs 3D, 5B). The acclimated males presented a significant change in EOD waveform in comparison to control males (Fig. 5B) of August/September (Mann–Whitney test, $P<0.05$, $n_1=4$, $n_2=5$) and October (Mann–Whitney test, $P<0.05$, $n_1=4$, $n_2=5$). Acclimated males presented a DP2/DP1 ratio of 1.51±0.17, which indicated a very lengthened second phase, similar to those of fish captured in December (breeding season), which was 1.52±0.32 (Fig. 3D).

Testes of fish captured in July showed an almost homogeneous population of spermatogonia. Control fish collected in September from the wild presented a gonadal profile that was slightly more advanced than in July, in which it was possible to observe spermatogonia and cysts of early spermatocytes (Fig. 6). Fish acclimated for 30 days at 28°C showed a striking change in the cell profile of the testes in experiments carried out during August (fish collected in July; see 'Accl.1' in Fig. 6) and during October (fish collected in September; see 'Accl.2' in Fig. 6). The number of spermatogonia decreased, and cysts with spermatocytes in different stages of development appeared in great numbers. In addition, a very significant increase in free spermatozoa was observed, grouped in large pools in the lumen. The overall aspect of the gonads was similar to those of breeding fish captured in November. Females also showed differences between control and experimental groups: ovaries were dominated by previtellogenic oocytes in control groups, whereas acclimated groups showed larger oocytes in different vitellogenic stages (data not shown).

Discussion

Gonadal histology

This is one of few studies of gonadal histology in Gymnotiformes. In particular, this is the first study to describe

the histological pattern for gonads of *Brachyhypopomus pinnicaudatus*. This species presents a saccular cystovary (Hoar, 1969). The presence of two or more oocyte types in each maturation stage, as well as the presence of post-ovulatory follicles in ovaries that still contained developing vitellogenic oocytes, indicate that *B. pinnicaudatus* is a multiple spawner (Blaxter and Hunter, 1982) and has a synchronous group ovary (Yamamoto and Yoshioka, 1964). Testicles are of the lobular type (Billard, 1990) and the presence of spermatogonia all along the testicular tubules characterizes them as of the 'unrestricted spermatogonial' type, as defined by Grier (1981). The presence of more than one spermatogenic stage in the testis confirms the previous conclusion that *B. pinnicaudatus* is a multiple spawner. There is good evidence that other Gymnotiformes such as *Eigenmannia virescens*, *Adontosternarchus* (Provenzano, 1984), *Gymnorhamphichtys hypostomus* (Schwassmann, 1976), and *Electrophorus* (Assunçao and Schwassmann, 1992) are all multiple spawners.

It is interesting to note that the gonads of *B. pinnicaudatus*, as those of all studied Gymnotiformes (Kirschbaum, 1995a), are located ventrally and exit (together with the intestine) at an extremely rostral position. This design is very rarely seen in teleosts (Kirschbaum, 1995a). As with other Gymnotiformes, this family presents an EO that runs from just behind the chin (ventral surface) to the tip of the caudal filament (Bennet, 1971). In ontogenetic studies in *B. pinnicaudatus* it has been observed that the anus and the anterior margin of the anal fin move rostrally during development (Franchina, 1997). These data suggest that the extension and location of the EO may have influenced a gonad displacement away from the most common location of teleost gonads.

Temperature as a zeitgeber of reproduction

Different environmental variables (temperature, photoperiod and conductivity) may modulate gonadal histology. Gymnotiformes have been mainly studied as tropical fish, and their reproductive cycles have been associated to the changes in conductivity related to the alternation of rainy and dry seasons (Kirschbaum, 1979). This study addresses the analysis of zeitgebers in a gymnotiform in the temperate region. It is well established that photoperiod and temperature are two environmental cues used by temperate-zone teleost fishes in the regulation of their reproductive cycles (Lam, 1983; Prosser and Heath, 1991; Kirschbaum, 2000). As expected in this region, temperature and photoperiod showed changes throughout the year whereas water conductivity was relatively low and constant (Silva et al., 2003).

In a previous study in a natural population of *B. pinnicaudatus* we made a preliminary identification of the breeding season from November to January, based upon data of population structure, external dimorphic morphological signs and EOD waveform (Silva et al., 2003). Our present results confirm this identification of the breeding season. There was a gradual maturation of germ cells towards November/December, and a clear temporal correlation between high water temperature, extreme photoperiod (10 h:14 h D:L) and the appearance of sexual maturity demonstrated by gonadal characteristics.

Gymnotiformes have been bred in captivity by putting fish in conditions that closely resemble the natural habitat (Kirschbaum, 1979, 1995a; Silva, 2002). Kirschbaum (1979) kept fish in large tanks, in groups of at least five, and manipulated many variables at once: conductivity, water level, and acoustic equivalent of rainfall. In Mormyrids that have been similarly bred in the laboratory (Kirschbaum, 1987, 1995a, 2000), captivity under regular conditions causes rapid and profound changes in the endocrine system that result in a dramatic decline of sexual maturity (Landsman, 1991, 1993). In this study, fish were subjected to captivity conditions that did not resemble the natural breeding habitat: social isolation, small individual tanks, constant day–night cycle (12 h:12 h), and low and constant water conductivity. Notably, we successfully induced gonadal recrudescence by manipulating water temperature alone.

Low temperature acclimation of mature *B. pinnicaudatus* provokes a decline in morphological and electrophysiological signs of sexual maturity (Silva et al., 1999). The caudal filament becomes thinner in males and their EODs become shorter, losing all male characteristics after 30 days (Silva et al., 1999). In the present study, non-differentiated fish kept in the high-temperature acclimation set-up underwent changes in gonad histology: both males and females were induced to mature. The gonads of fish after acclimation were very similar to those captured from the wild in November, during the breeding season; spawning was not achieved in captivity under these conditions.

High water temperature is sufficient to trigger gonadal maturity and therefore confirms its role as a zeitgeber of reproduction of *B. pinnicaudatus* in the temperate region. In the temperate region there are two main environmental factors that change seasonally (temperature and photoperiod); our results allowed us to conclude that photoperiod is not necessary for the onset of sexual maturity. Nevertheless, its influence upon the reproduction of this species cannot be ruled out.

The precise route by which environmental factors influence the neuroendocrine system has not been unraveled so far. In cyprinids, temperature is the main environmental factor: warm temperatures cause an increase of basal gonadotrophin release and of pituitary responsiveness to the hypothalamic factor GnRH, which in turn induces gonadal recrudescence (high gonadosomatic index and testosterone levels) (Hontela and Peter, 1978; Breton et al., 1980a,b; Peter, 1981; Razani et al., 1988a,b; Lin et al., 1996).

Electrophysiological sexual maturity indicators

Gonadal maturation obviously implies the secretion of steroid hormones, which play a major role in mediating reproductive behavior, either by acting directly on brain structures governing certain behavior patterns or by acting indirectly to influence behavior through their effects on the development of secondary sexual characteristics (Liley and Stacey, 1983). In Gymnotiformes, several studies have

demonstrated that different parts of the electrogenic system are targets of steroid hormones. For example, the sexual dimorphism of EOD waveform, a peripheral phenomenon, has been shown to be causally linked to naturally occurring or experimentally manipulated levels of sex steroid hormones (Zakon, 1998; Zakon et al., 1999). In addition, the emission of social signals during breeding behavior, governed by certain brain nuclei, is also modulated by steroid hormones (Dulka and Maler, 1994; Dulka et al., 1995; Dulka and Ebling, 1999; Dunlap and Zakon, 1998; Dunlap, 2002). In *B. pinnicaudatus*, electric sexual dimorphism and temperature sensitivity are driven by steroid hormones and have been postulated as sexual maturity indicators (Silva et al., 2002). In this study hormone measurements were not carried out, but these two maturity indicators were measured in the wild and experimentally modulated.

Male electrophysiological dimorphism showed an annual cycle and was evident during the breeding season. This electrophysiological feature coincided, as expected, with the appearance of gonadal maturity, as well as with the typical breeding habitat environmental conditions (high water temperature and extreme photoperiod). This EOD dimorphism was successfully induced in acclimated males. These fish, placed for 1 month at a high and sustained temperature, significantly lengthened the P2 phase of their EOD. On the other hand, low temperature acclimation of sexually mature *B. pinnnicaudatus* induced shortening of their characteristically lengthened P2 phase after 15 days (Silva et al., 1999). Captivity, regardless of housing conditions, exerts similar effects in Mormyrids: EOD sexual identity is rapidly lost in relation to a decay of androgen levels (Landsman, 1991, 1993; Landsmann, 1995). We have, through 28°C-acclimation, achieved effects similar to those of androgen treatments (Silva et al., 1999). Our results reinforce the idea that acclimation affects hormone levels which account for sexual dimorphism.

EOD temperature sensitivity is probably a widespread phenomenon, and has been described so far in *B. pinnicaudatus* and *Gymnotus carapo* (Caputi et al., 1998; Silva et al., 1999; Ardanaz et al., 2001; Silva et al., 2002). EOD temperature sensitivity showed annual changes: it was high during the non-breeding season and practically null during breeding; moreover, it was negatively correlated to gonadal stages. Accordingly, juveniles show very high temperature sensitivity all year round, including summer (Silva et al., 1999). Previous studies have also demonstrated that acclimation and androgen treatment induce a decrease in temperature sensitivity in both *B. pinnicaudatus* and *G. carapo* (Silva et al., 1999, 2002; Ardanaz et al., 2001). In the same sense, sexually mature *B. pinnicaudatus* increase their temperature sensitivity when acclimated at 20°C during 30 days, and decrease it when later acclimated at a high temperature (Silva et al., 1999). The decrease in EOD temperature sensitivity, observed during the breeding season, may be interpreted as a way of protecting the communication value of the second phase of the EOD, thus allowing this lengthened phase to be a reliable and detectable sign of reproductive state (in otherwise unfavorable conditions).

This study provides crucial evidence to prove that high water temperature is sufficient to induce gonadal maturity in *B. pinnicaudatus*, probably acting through the hypothalamus–hypophysial axis. In addition, temperature also induces male EOD dimorphism and a decrease in temperature sensitivity, two electrical phenomena which emerge as a direct consequence of gonadal maturity.

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