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Reproductive and parental care physiology of *Cichlasoma dimerus* males

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ABSTRACT

The South American cichlid fish *Cichlasoma dimerus* presents a high breeding frequency and biparental care of the eggs and larvae. The male parental care period was divided in four different phases according to the developmental degree of the offspring: pre-spawning activity (MP, day 0), guarding eggs (ME, one day after fertilization (1 DAF)), guarding hatched larvae (MHL, 3 DAF), and guarding swimming larvae (MSL, 8 DAF). The aim of this study was to characterize male reproductive physiology by measuring steroid hormone plasma levels and analyzing testes cellular composition. Males exhibiting pre-spawning activity showed 8.4 times higher 11-ketotestosterone and 5.63 times higher testosterone levels than MHL. No differences were observed in estradiol and cortisol levels among the different phases. The cellular composition of the testes varied during the reproductive and parental care periods. Testes of MP were composed of 50% of spermatozoa, whereas spermatogonia type B and spermatocytes were predominant in the subsequent parental phases. A morphometric analysis of Leydig cells nuclear area revealed that MP and ME's Leydig cells averaged 1.27 times larger than that those of MHL and MSL and was positively correlated with circulating 11-KT and T levels. Hence, *C. dimerus* males showed important changes in its hormonal profiles and testicular cellular composition throughout the reproductive and parental care period.

1. Introduction

Parental care represents a trade-off between the survival of the offspring and parents' future reproduction (Huxley, 1938). This trade-off is particularly strong for males, whose energy investment in gamete production is lower than that of females (Bateman, 1948). Trivers (1974) defined parental care as any form of parental behavior that appears likely to increase the fitness of the offspring. Within the animal kingdom, fishes show the greatest variety of parental care, especially within cichlids (Goodwin et al., 1998). Fish parental behavior includes activities such as nest construction, tending, guarding and fanning of the eggs and guarding of the brood from predators (Royle et al., 2012). The form of parental care is strongly related to the mating system. Fish species with parental care and lek-breeding or harem systems for example, show a strong association with maternal mouthbrooding; such is the case for *Oreochromis* sp. and *Astatotilapia burtoni* (Fernald and Hirata, 1977; Turner and Robinson, 2000).

The endocrine system plays an important role in the control of reproductive and parental behavior in vertebrates (Gans, 1996; Knapp et al., 1999; Reburn and Wynne-Edwards, 1999; Slater and Milinski, 1996). It is generally found that androgen levels, such as 11-ketotestosterone (11-KT) and testosterone (T), decrease during parental care periods because of an apparent incompatibility of male parental behaviors with aggression (reviewed by Hirschenhauser et al., 2003). However, it was demonstrated in *Neolamprologus pulcher* (Desjardins et al., 2008), *Gasterosteus aculeatus* (Páll et al., 2002), *Lythrypnus dalli* (Rodgers et al., 2006) and *Parablennius parvicornis* (Ros et al., 2004) that elevated androgen levels do not necessarily decrease parental investment. Moreover, in the male bluegill sunfish (*Lepomis macrochirus*) no androgen-mediated trade-off appeared to exist between parental aggression and nurturing behavior (Rodgers et al., 2012). Furthermore, males

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of cichlid species with exclusive maternal care have lower plasma androgen levels than males of closely related cichlid species with biparental care (Hirschenhauser et al., 2004).

Another sex steroid involved in the regulation of aggression and reproductive physiology is estradiol (E2) (Huffman et al., 2013; Kishida and Specker, 2000; Specker and Kishida, 2000). It was shown that the enzyme aromatase promotes aggression in dominant males of *A. burtoni* by the conversion of T into E2; however, the inhibition of aromatase activity did not affect reproductive behavior (Huffman et al., 2013). On the other hand, in *Sarotherodon melanotheron* evidence suggests a negative relationship of male parental behavior with plasma E2 levels (Specker and Kishida, 2000).

In addition, stress may play an important role in reproduction and parental care (Knapp et al., 1999; Milla et al., 2009). Secretion of the glucocorticoid cortisol (CORT) is a primary indicator of stress in teleosts (Pankhurst, 2011), and its levels have been found to fluctuate across breeding stages, with a peak soon after spawning (Magee et al., 2006; Tubert et al., 2012), suggesting the care of eggs as a stressful event. However, high CORT levels have also been linked to an increase in nest failure (Dey et al., 2010; Magee et al., 2006; O'Connor et al., 2009). Therefore, the role of CORT in parental care is not clear.

The South American cichlid fish *Cichlasoma dimerus* (Heckel, 1840) is a well-established laboratory model for the study of reproduction, neuroendocrinology and behavior (reviewed by Ramallo et al., 2014). This species breeds at high frequency and exhibits biparental care of eggs and larvae that extends up to 20 days post-spawning (Tubert et al., 2012). The cooperative pair takes care of the eggs, both by fanning them and by removing dead ones. After hatching of the eggs, larvae are transferred by both parents to a previously dug pit (Meijide and Guerrero, 2000). Finally, the pair takes care of the swimming brood, guarding them from predators. The parental care period can be divided in four different phases according to the offspring's stage of development: male showing prespawning activity (MP, day 0), male guarding eggs (ME, one day after fertilization (1 DAF)), male guarding hatched larvae (MHL, 3 DAF), and male guarding swimming larvae (MSL, 8 DAF).

Until now, male *C. dimerus* reproductive physiology was only described to vary between reproductive and non-reproductive stages (Rey Vázquez et al., 2012), while the present work focuses on the less studied variation within the reproductive period. In this paper, we aimed to assess the reproductive and parental care physiology in male *C. dimerus* through the analysis of plasma steroid levels and cellular composition of the testes during the four phases of the parental care period.

2. Materials and methods

2.1. Animals

Male and female adult specimens of *C. dimerus* were collected from wild populations in Esteros del Riachuelo (27°35′S; 58°45′W; Corrientes, Argentina). Fish were then transferred to our laboratory in Buenos Aires and housed in community tanks (150 L, 6–8 fish per tank) with aquatic plants and stones, under conditions mimicking their natural reproductive habitat: 25 ± 2 °C and 14:10 light:dark cycle with full spectrum illumination, external filtration and constant aeration. Every morning, fish were fed *ad libitum* with fish food sticks (Koi Vibrance Color Enhancer Fish Food, Tetra Brand®). Animals were allowed to acclimate to aquarium conditions for at least one month before their incorporation into the experimental set up.

All experiments were conducted in accordance with international standards on animal welfare (Guide for the care and use of laboratory animals, 2011), and were previously approved by the local Ethical Committee (CICUAL, Facultad de Ciencias Exactas y Naturales, Universidad de Buenos Aires).

2.2. Experimental design

We obtained reproductive pre-spawning pairs from community tanks containing 6–8 fish (150 L; temperature 25 ± 2 °C and 14:10 light:dark cycle with full spectrum illumination). Each pair was then isolated in a smaller tank (75 L) with a flat slab at the bottom of the aquarium on which fish usually spawned. The male parental care stage was divided in the four aforementioned phases: prespawning activity (MP, day 0), guarding of fertilized eggs (ME, 1 DAF), guarding of hatched larvae (MHL, 3 DAF), and guarding of swimming larvae (MSL, 8 DAF). In total, 25 pairs were isolated.

Males from pairs in each of the four phases (mean body mass: 39.9 ± 4.4 g; total length: 12.7 ± 0.8) were removed from the isolated aquaria, and we immediately drew blood by puncture of the caudal vein with heparin-coated syringes (needle: 27 gauge × 1/2 inch) and collected it in heparin-coated tubes. To minimize possible effects of circadian variation, all samples were taken between 14:00 and 15:00 h. Blood was kept overnight at 4 °C and centrifuged at 3000 rpm for 15 min. Finally, the plasma was collected and stored at −20 °C until assayed.

After blood collection, males from each reproductive phase were anesthetized by immersion in a 0.1% benzocaine solution until opercular movement ceased. Body mass, total and standard length of each animal were recorded. The pair was then euthanized by decapitation. The testes were rapidly dissected and weighed for calculation of the gonad-somatic index (GSI: [gonad mass/total body mass] × 100). One testis from each fish was fixed in Bouin's solution for 16–18 h for histological processing. In order to further understand the reproductive dynamic in males of this species with multiple spawning events, we performed a karyometric analysis of Leydig cells as an indicator of testicular steroidogenic activity, as well as an analysis of the preponderance of each spermatogenic cell type.

2.3. Hormone assays

Steroid hormones (11-KT, T, E2, and CORT) were measured from plasma samples using commercial ELISA kits. As CORT may quickly increase due to fish manipulation (Fox et al., 1997), it was only quantified from blood samples for which drawing time was less than 4 min after netting. Working dilutions were 1:6 for 11-KT (some samples from pre-spawning males had to be diluted even further, up to 1:50, as 11-KT concentration was above the upper curve limits), 1:2 for T and E2 and undiluted for CORT. In all cases, samples were assayed in duplicate and analyses were carried on samples for which coefficients of variation were below 20%, following the manufacturer's instructions. Intra-assay variation was 11.1% for 11-KT (detection limit: 1.3 pg/ml; Cayman Chemical Company, MI, USA); 10.6% for T (detection limit: 0.07 ng/ml; IBL International, Hamburg, Germany); 18.6% for E2 (detection limit: 9.7 pg/ml; IBL International, Hamburg, Germany), and 8.6% for CORT (detection limit: 2.46 ng/ml; IBL International, Hamburg, Germany), while inter-assay variation was 5.4%, 15.6%, 5.3% and 10.3% respectively. Parallelism to standard curves was assessed by measuring hormone concentration of serially diluted samples (4 different dilutions). Correlation coefficients were 0.99 for 11-KT, 0.99 for testosterone, 0.98 for E2 and 0.97 for CORT after log transformation.

2.4. Quantification of testicular cell types

After fixation, testes were dehydrated through an ascending series of ethanol, clarified with xylene, embedded in Paraplast®, serially sectioned at 7 μm, and stained with Masson's Trichrome.

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Three randomly chosen sections, separated by at least five sections, from each fish testis were then examined with a Microphot FX (Nikon) microscope and digitally photographed (Coolpix 4500, Nikon).

To quantify the testicular cell types, 60 randomly generated points were overlaid on each photomicrograph using CPCe software® and the cell type beneath each cross-hair point was identified according to Rey Vázquez et al. (2012). In a pilot study, 60 points proved sufficient to adequately determine the percentage of all cell types (45, 60, 75 and 90 points were tested; $p > 0.05$ for 60, 75 and 90 points). Quantified cell types were: type A and B spermatogonia (SG A; SG B), spermatocytes (SC), spermatids (SD), spermatozoa (SZ) and interstitial tissue (IT). Percentages for each cell type were then averaged from the three photomicrographs.

2.5. Morphometric analysis of Leydig cells' nucleus

Leydig cells are the main source of gonadal steroids in male fish (Nagahama et al., 1982). Nuclear area was analyzed as an indirect measurement of overall cellular activity. An increase in protein synthesis for steroid production may be accompanied by a larger nuclear area, which has been used as a reliable indicator of cellular activity in fish (Cerdá-reverter et al., 2001; Piazza et al., 2011). The nuclear areas were computed from digital images of Leydig cells at 600× with the software Image Pro Plus 4.5 (Media Cybernetics) which was previously calibrated with a stage micrometer. All nuclear areas (μm^2) were measured by tracing the cell's nucleus profile with a digitizing pen. We measured 30 randomly chosen Leydig cells with a clear nucleus from each testis.

2.6. Data analysis

All statistical analyses were performed using Statistica 8 (StatSoft®). All data fulfilled the criteria for parametric statistics. Gonado-somatic indexes, cellular composition of the testes, plasma steroid levels and the morphometric data from Leydig cells were compared by one-way analysis of variance (ANOVA). The preponderance of cell types in each reproductive and parental care phase was analyzed by ANOVA with split plot design. We investigated whether there was any correlation between plasma levels of T or 11-KT, and the nuclear area of Leydig cells. Data are presented as mean ± SEM. When significant differences were found, the analyses were followed by Tukey test.

3. Results

3.1. Plasma steroid profiles throughout the phases of parental care period

The distinct reproductive phases were accompanied by different hormonal profiles. Circulating 11-KT levels varied between the four phases ($F = 5.87$, $p = 0.004$). Males with pre-spawning activity had on average 8.4 times higher 11-KT plasma levels than MHL (MP: 1.96 ± 0.58 ng/ml; MHL: 0.23 ± 0.14 ng/ml; $p = 0.014$), 13.5 times higher than MSL (MSL: 0.11 ± 0.04 ng/ml; $p = 0.018$) (Fig. 1a). Circulating levels of T also varied among the four phases, MP had on average 5.6 times higher T than MHL (MP: 4.07 ± 1.30 ng/ml; MHL: 0.72 ± 0.25 ng/ml; $F = 4.06$; $p = 0.02$) (Fig. 1b). Estradiol and CORT plasma levels did not show differences between the reproductive and parental care phases (E2: $F = 2.05$, $p = 0.14$ and CORT: $F = 0.18$, $p = 0.96$) (Fig. 1c and d, respectively).

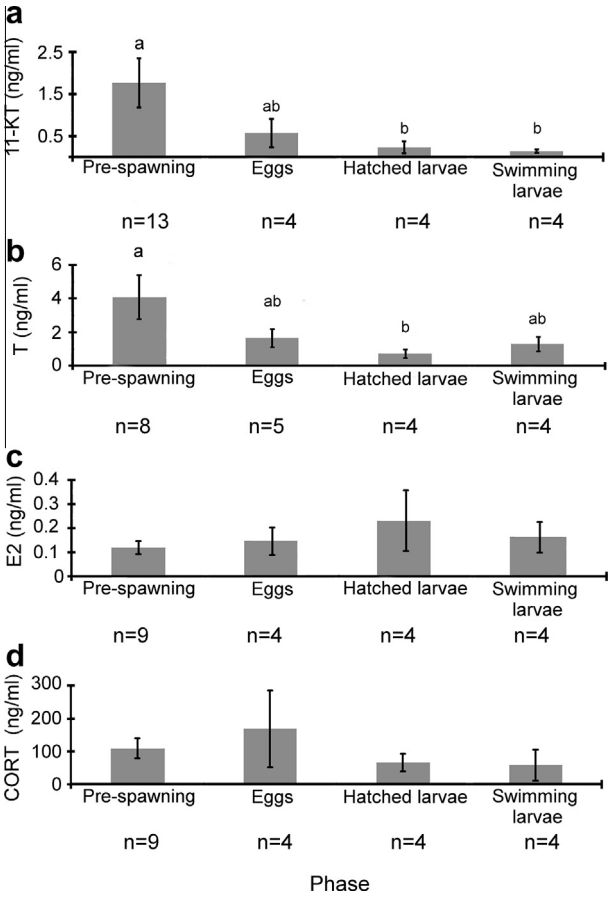

Fig. 1. Plasma steroid profiles throughout the four phases of the reproductive and parental care period. Plasma levels of (a) 11-ketotestosterone (11-KT), (b) testosterone (T), (c) estradiol (E2) and (d) cortisol (CORT) from males in the four different phases. Different letters indicate significant differences. Data are presented as mean ± SEM.

3.2. Gonado-somatic index throughout the phases of parental care period

No difference in GSI was found among males from the four phases (MP = 0.10 ± 0.01%, $N = 10$; ME = 0.11 ± 0.02%, $N = 5$; MHL = 0.08 ± 0.03%, $N = 5$; MSL = 0.09 ± 0.04%, $N = 5$ $F = 0.11$; $p = 0.95$).

3.3. Cellular composition of the testes throughout the phases of parental care

3.3.1. Qualitative and histological description of the testes

Testes from MP contained mainly later stages of spermatogenesis with a strong preponderance of spermatozoa. The mature testes' lobules had lumens filled with sperm (Fig. 2a), while the interstitial tissue occupied a smaller area (Fig. 2a). Moreover, the lobular lumen was minimal compare to the rest of the phases (Fig. 2a and b).

After spermiation, the lumen of ME testes contained residual sperm within the lobules and the interstitial compartment was more noticeable than in MP. In ME there was a preponderance of SG B and SC (Fig. 2b).

Testes from MHL showed scarce sperm within the lobules' lumen. There was a preponderance of spermatocytes in different stages of the first meiotic division (Fig. 2c).

Finally, testes from MSL exhibited a prevalence of sperm within the lobular lumen (Fig. 2d).

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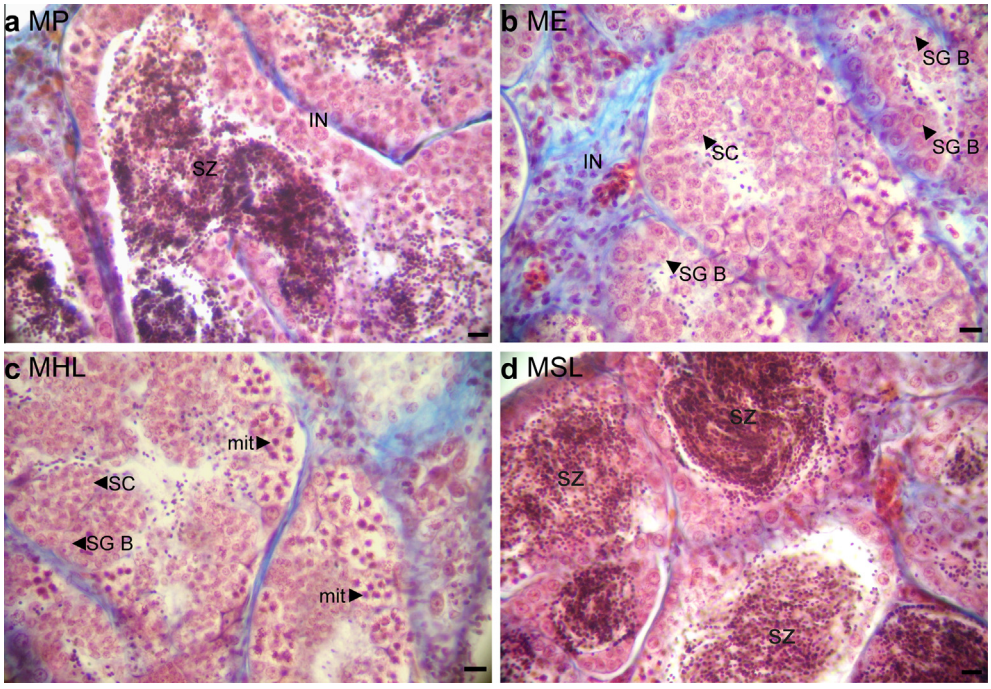

Fig. 2. Histological cross sections of *C. dimerus* testes during the reproductive and parental care phases. (a) Detail of a testicular lobule from a male exhibiting pre-spawning activity (MP with a preponderance of mature sperm). (b) Detail of a testicular lobule from a male guarding eggs (ME) with a preponderance of spermatogonia type B and spermatocytes. Within the testicular lumen there is residual sperm. (c) Detail of a testicular lobule from male guarding hatched larvae (MHL), with a preponderance of spermatogonia type B. Many of the spermatogonia type B or the spermatocytes present mitotic figures. (d) Detail of a testicular lobule from male guarding swimming larvae (MSL) with abundant mature sperm within the testicular lumen. IN: interstitial tissue; mit: mitotic figures; SC: spermatocytes; SG B: spermatogonia type B; SZ: spermatozoa. Histological staining technique: Masson's Trichrome stain; scale bars: 10 μm.

3.3.2. Quantitative analysis of the testes

Males exhibiting pre-spawning behavior had testes primarily comprised of mature sperm within the testicular lumen (51.8 ± 4.0%; $p < 0.05$) (Fig. 3a). Males with eggs showed a preponderance of SG B and SC (31.9 ± 5.2% and 35.9 ± 3.7%, respectively; $p < 0.05$) (Fig. 3b). During the MHL phase, a predominance of SG B could be observed (37.9 ± 3.9%; $p = 0.0008$) (Fig. 3c). Finally MSL showed a preponderance of spermatocytes (30.4 ± 3.5, $p = 0.002$) (Fig. 3d).

The cellular composition of the testes varied between the four phases of the parental care period ($F = 2.48$; $p = 0.031$; $N = 4$ for each reproductive phase). Males with pre-spawning activity showed the highest percentage of spermatozoa (51.8 ± 4.0%; $p < 0.05$) (Fig. 4e); whereas SG and SC were predominant in the subsequent phases. Spermatogonia type B percentage was highest in MHL, representing 37.9 ± 3.9% of the testis's cellular composition (Fig. 4b). Males guarding hatched larvae presented 23% more SG B than MP (14.0 ± 0.9%; $p = 0.02$) and 13% more than MSL (19.2 ± 2.5%; $p = 0.048$) (Fig. 4b). Furthermore, ME had 18% more SG B and SC than MP (SG B: 31.9 ± 5.3%, $p = 0.048$; SC: 35.9 ± 3.1%, $p = 0.041$) (Fig. 4b and c). The percentage of SG A, SD and IT did not vary among the phases (Fig. 4a, d and f).

3.4. Morphometric analysis of Leydig cells throughout the phases of parental care period

Leydig cells' nuclear area was on average 1.27 times larger in the first two phases of the reproductive cycle compared to the last two (MP = 16.0 ± 0.6 μm^2; ME = 14.4 ± 0.3 μm^2; MHL = 11.5 ± 0.5 μm^2; MSL = 12.4 ± 1.0 μm^2; $F = 8.4$; $p = 0.0042$; $N = 4$ for each reproductive phase) (Fig. 5a).

Leydig cell nuclear area correlated positively with plasma 11-KT and T levels, after combining the data from all types of males. An exponential fit ($[11\text{-KT}] = \exp^{\beta \cdot \text{Leydig cell nuclear area}}$) best explained the relationship between 11-KT and Leydig cell nuclear area ($\beta = 0.6 \pm 0.1$; $p = 0.001$; $\alpha = 0.05$) (Fig. 5b), while the relationship was linear for T ($r = 0.82$; $p < 0.0001$) (Fig. 5c).

4. Discussion

In the present work, we describe *C. dimerus* paternal physiology, by analyzing testicular and hormonal profiles during the reproductive and parental care period. The experimental design allowed us to study hormonal and testicular variations through the reproductive and parental care period, by dividing it in four phases according to the offspring's stage of development: MP, ME, MHL and MSL.

Several hormones are involved in the modulation of specific behavioral patterns in fish. Results obtained in this work showed that MP have the highest concentration of plasma 11-KT and T. This result is in concordance with those observed by Tubert et al. (2012), where pre-spawning *C. dimerus* females showed the highest plasma androgen levels. Therefore, both females and males present high androgen levels at the pre-spawning phase. This may be related to the performance of aggressive behaviors, strongly associated to the establishment of a hierarchical dominance, characteristics of *C. dimerus* (Alonso et al., 2011, 2012), and the defense of the spawning territory, in the community tank before being isolated. However, in the Mozambique tilapia (*Oreochromis mossambicus*), castrated males showed low plasma androgen levels and no reproductive behavior, but still performed aggressive behaviors (Almeida et al., 2014). Hence, the elevated androgen levels observed in male and females *C. dimerus* with pre-spawning activity may be related to reproductive behaviors *per se*, and not with the establishment of the hierarchical dominance and the defense of the spawning territory.

In *C. dimerus* males, androgen levels decreased across reproductive phases, with intermediate 11-KT and T values while guarding the eggs. This result is opposite to that observed in male bluegill sunfish, where androgen plasma levels typically sharply decrease

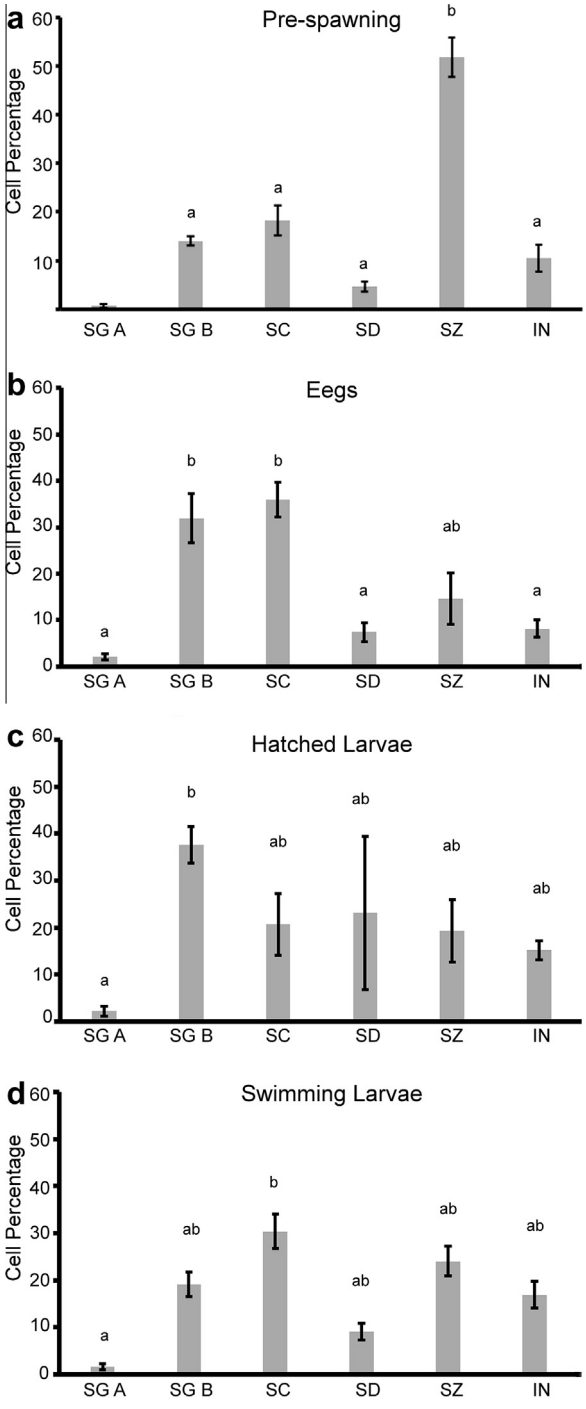

Fig. 3. Testicular dynamics throughout the reproductive and parental care phases. (a) Cellular composition of the testis from males with pre-spawning activity, where a preponderance of mature spermatozoa was detected. (b) Cellular composition of the testis from males guarding eggs, where spermatogonia type B and spermatocytes predominate. (c) Cellular composition of the testis from males guarding hatched larvae. A peak of spermatogonia type B can be observed. (d) Cellular composition of the testis from males guarding swimming larvae showing an even representation of the different stages of spermatogenesis. SG A: spermatogonia type A; SG B: spermatogonia type B; SC: spermatocytes; SD: spermatids; SZ: spermatozoa. Different letters indicate significant differences. Data is presented as mean percentage ± SEM. $N = 4$ for each phase.

during spawning and remain low through the following two days (Magee et al., 2006). A similar pattern to that observed in *C. dimerus* was present in the plainfin midshipman (*Porichthys notatus*), in which high plasma androgens levels were sustained in both pre-spawning and post spawning periods (Knapp et al., 1999). Our data suggest that elevated androgen levels are compatible with parental care behavior, at least to some degree, as plasma 11-KT and T levels of ME were comparable to that of MP. Plasma 11-KT and T levels observed in MHL and MSL are higher than those of non-territorial *C. dimerus* males, which are socially denied access to reproduction, and the latter is also physiologically suppressed. This non-territorial males show lower 11-KT and T levels compared with territorial males (Morandini et al., 2014). At all four phases of the parental care period, levels of both evaluated androgens are higher than those present in the suppressed non-territorial males, suggesting an active maintenance of reproduction. Furthermore, the impact of elevated androgen levels on parental care has been examined in several studies and it has been repeatedly demonstrated that high androgen levels do not necessarily decrease parental investment in male fish (Desjardins et al., 2008; Hirschenhauser et al., 2004; Páll et al., 2002; Ros et al., 2004; Rodgers et al., 2006).

In *C. dimerus* males plasma E2 levels remained constant during the four phases of the reproductive and parental care period. This result is consistent with that observed for other species of non mouthbrooding fish where plasma E2 levels were not detectable or shown not to vary (Fostier et al., 1983; Kadmon et al., 1985; Knapp et al., 1999; Magee et al., 2006; Rosenblum et al., 1987). On the other hand, in females, E2 plasma levels were seen to vary throughout the reproductive cycle, with an increase before spawning (*Fundulus heteroclitus*, Cerdá et al., 1996; *Gadus morhua*, Dahle et al., 2003; *C. dimerus*, Tubert et al., 2012). Therefore, E2 seems to be necessary in female's reproductive period, though not in that of males. This is consistent with the fact that E2 is involved in the control of the synthesis of vitellogenin (see reviews Mommsen and Walsh, 1988).

The major product of the fish interrenal gland is CORT (Mommsen et al., 1999), whose plasma levels increase during stress. In *C. dimerus* females it was shown that plasma CORT levels dramatically increase during guarding of the eggs, and then decline and remain low in the subsequent phases. In the present work, we found that *C. dimerus* males' CORT levels did not vary during the four phases of the reproductive period. Cortisol levels were comparable to those observed in territorial *C. dimerus* males, which in turn were considerably lower than those of non-territorial males (Morandini et al., 2014). This result is in agreement with some studies in largemouth bass (*Micropterus salmoides*) where plasma CORT levels were manipulated, and seemed not to affect the intensity of parental care behavior (O'Connor et al., 2009).

The gonadosomatic index is a common metric used to estimate reproductive investment in fishes, but in males it does not provide information on more subtle cellular changes that can have important functional consequences for sperm and steroid production (Maruska et al., 1996). No differences were found between males' GSI from the four phases. Therefore, we further analyzed the testes cellular composition throughout the reproductive and parental care period and characterized the proportion of each cellular type during the four phases. Males exhibiting pre-spawning activity presented testes composed of 51.8 ± 4% mature spermatozoa. After spawning, ME exhibited an elevated mean percentage of SG B and SC (31.9 ± 5.2%; 35.9 ± 3.1, respectively) and MHL had an elevated mean percentage of SG B (37.9 ± 3.9%), reflecting a high level of germ cell proliferation. Finally, testes from MSL showed a more homogenous distribution of each cell type with a preponderance of SC. Thus, *C. dimerus* spermatogenesis remains active during the parental care periods, which is in concordance with the fact that *C. dimerus* undergoes multiple spawning events within a single reproductive period (November–March) (Rey Vázquez et al., 2012).

In this work, we showed that MP and ME *C. dimerus* exhibited and increased steroidogenic activity of their Leydig cells, as

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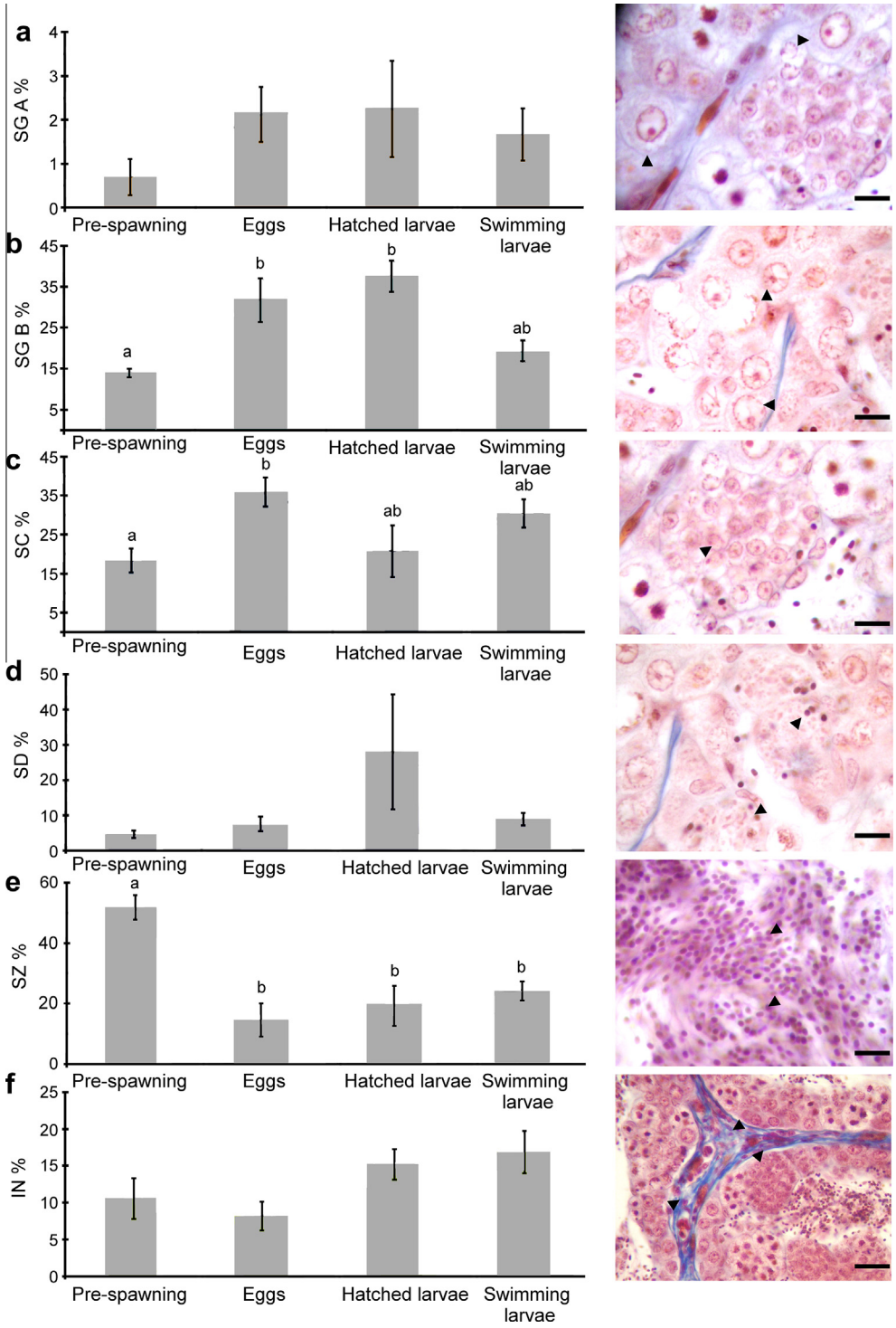

Fig. 4. Percentage of each cell type throughout the four phases of the reproductive and parental care phases. (a) SG A%: spermatogonia type A percentage; (b) SG B%: spermatogonia type B percentage; (c) SC%: spermatocytes percentage; (d) SD%: spermatids percentage; (e) SZ%: spermatozoa percentages; (f) IN%: interstitial tissue percentage. Representative photomicrographs of each cell type are shown at the right of each corresponding graph; arrowheads indicate the cell type that was quantified. Different letters indicate significant differences. Data are presented as mean percentage ± SEM. MP: males with prespawning activity; ME: males guarding eggs; MHL: males guarding hatched larvae; MSL: males guarding swimming larvae. $N = 4$ for each phase. Masson's histological staining technique: Trichrome stain; scale bar: 10 µm.

estimated through the measurement of cells' nuclear area. Moreover, MP exhibited higher plasma levels of 11-KT and T, compared to MHL. Androgens are effective in supporting either the whole process of spermatogenesis, or at least some steps such as spermatogonial multiplication and spermatocyte formation or maturation (Billard et al., 1982; Billard, 1986; Borg, 1994; Fostier et al., 1983; Nagahama, 1994). It has been shown that 11-KT and T strongly influence testicular gene expression (Le Gac et al., 2008; Rolland et al., 2011). For instance, expression of the anti-Müllerian hormone gene that inhibits the differentiation of

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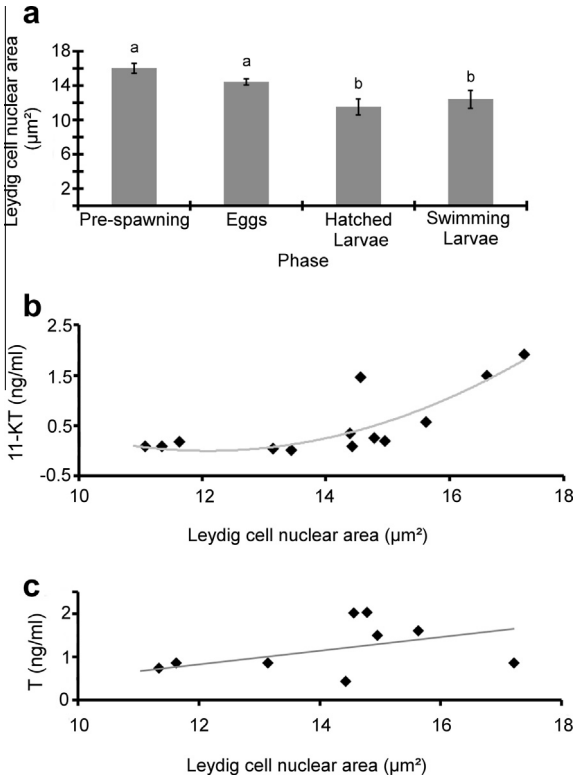

Fig. 5. Analysis of Leydig cells' nuclear area throughout the male reproductive and parental care phases. (a) Nuclear area of Leydig cells of *C. dimerus* males throughout the reproductive stage (N = 4 for each phase). Relationship between the nuclear area of Leydig cells and plasma levels of (b) 11-ketotestosterone (11-KT) (exponential fit; $\beta = 0.6 \pm 0.1$; $p = 0.001$; pre-spawning $N = 4$; eggs $N = 4$; hatched larvae $N = 3$; swimming larvae $N = 3$) and (c) testosterone (T) levels (Pearson coefficient; $r = 0.082$; $p < 0.0001$; pre-spawning $N = 3$; eggs = 2; hatched larvae $N = 3$; swimming larvae $N = 2$). Different letters indicate significant differences. Data is presented as mean ± SEM.

spermatogonia is significantly suppressed by 11-KT and T (Schulz et al., 2010). This is consistent with the higher plasma 11-KT and T levels observed in MP, and declining, but still measurable levels in the next phases, accompanying active spermatogenesis as fish "gear up" for the following reproductive event.

The morphometry of Leydig cells may be useful as an additional indicator of androgen production. One significant finding of our study is that the correlation between 11-KT plasma concentration and Leydig cells nuclear area best fitted to an exponential relationship rather than to a linear one, whereas the relation with T is linear. This could be explained by the fact that T mediates the enhancement of enzymatic activity and gene expression of 5α-reductase, which converts T to 5α-dihydrotestosterone, a precursor of 11-KT (Cohen and Wade, 2010). Therefore, Leydig cell synthesizes T and then when a significant concentration of T is accumulated, the conversion to 11-KT is accelerated. However, further studies are necessary to confirm this possibility.

5. Conclusions

C. dimerus males exhibiting pre-spawning activity presented the highest 11-KT and T plasma levels, Leydig cells with a larger nuclear area indicative of high steroidogenic activity and testes composed mainly of spermatozoa. In the later parental care phases, androgen levels were lower and testes showed a more homogenous distribution of spermatogenic cells. This evidence indicates active spermatogenesis as the fish quickly "gears up" for the next reproductive event.

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