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Aggressive behavior and reproductive physiology in females of the social cichlid fish *Cichlasoma dimerus*

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ABSTRACT

The South American cichlid fish *Cichlasoma dimerus* is a freshwater species that presents social hierarchies, a highly organized breeding activity, biparental care and a high frequency of spawning. Spawning is followed by a period of parental care (about 20 days in aquaria conditions) during which the cooperative pair takes care of the eggs, both by fanning them and by removing dead ones. The different spawning events in the reproductive period were classified as female reproductive stages which can be subdivided in four phases, according to their offspring degree of development: (1) female with prespawning activity (day 0), (2) female with eggs (day 1 after fertilization), (3) female with hatched larvae (day 3 after fertilization) and (4) female with swimming larvae (FSL, day 8 after fertilization). In Perciform species gonadotropin-releasing hormone type-3 (GnRH3) neurons are associated with the olfactory bulbs acting as a potent neuromodulator of reproductive behaviors in males. The aim of this study is to characterize the GnRH3 neuronal system in females of *C. dimerus* in relation with aggressive behavior and reproductive physiology during different phases of the reproductive period. Females with prespawning activity were the most aggressive ones showing GnRH-3 neurons with bigger nuclear and somatic area and higher optical density than the others. They also presented the highest levels of plasma androgen and estradiol and maximum gonadosomatic indexes. These results provide information about the regulation and functioning of hypothalamus–pituitary–gonads axis during reproduction in a species with highly organized breeding activity.

1. Introduction

Aggression is a common behavior in the context of competition for limited resources such as food or mating [1]. The behavior observed during the reproductive season, either defending a territory, during breeding, or during the protection of the offspring, is critical for the reproductive success.

The South American cichlid fish *Cichlasoma dimerus* is a freshwater species that presents social hierarchies, a highly organized breeding activity, biparental care and a high frequency of spawning. The dominant pair will strongly defend the prospective spawning site. Spawning is followed by a period of parental care (about 20 days in aquaria conditions) during which the cooperative pair take care of the eggs, both by fanning them and by removing dead ones. At a 26 °C water temperature larvae hatch at the beginning of the third day after fertilization (DAF) and are transferred by both parents to a previously dug pit. Larvae spend five days in the pit until they swim freely [2]. Aggressive behaviors include biting, mouth holding, chasing, fin erection, while submissive displays include escape and fin retraction [3].

Gonadotropin-releasing hormone type-3 (GnRH3) neurons are associated with the olfactory bulbs and they project axons to the retina and pineal organ which suggest a role in the light and photoperiodic behavioral and physiological responses [4,5]. In male tilapia *Orechromis niloticus* it was shown that GnRH3-immunoneutralization significantly decreases nest-building ability, nest size and aggressive behavior [6]. In male dwarf gourami *Colisa lalia* GnRH3 modulates reproductive behaviors related to nest building [7]. It has been proposed that GnRH3 could be acting on the visual and olfactory system coordinating sensory inputs with reproductive requirements [8]. Those results provided evidence that GnRH3 is a potent neuromodulator of reproductive behaviors in males, but no studies have been performed in females.

Aggression has also been associated with androgen levels. Manipulation experiments have demonstrated that androgen removal decreases aggression while androgen treatment rescues or increases aggression [9–11]. In the male cichlid *Orechromis mossambicus*, androgen treatment increases aggression and territorial defense [12]. These observations, paired with demonstrations that individuals

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with high androgen levels exhibit high levels of aggression [13] have led the scientists accept circulating androgens as potent mediators of male aggressive behavior [14]. More recently, both male and female aggression and androgen levels have been strongly associated with dominance over conspecifics [15,16]. Also, it has been demonstrated that in the highly social cichlid *Neolamprologus pulcher*, dominant females have higher testosterone but not 11-ketotestosterone than subordinate females, while dominant males have higher 11-ketotestosterone but not testosterone than subordinated males [14].

In order to better understand the HPG axis during reproduction in species with highly organized breeding activity, the aim of this study is to characterize the GnRH3 neuronal system in females of *C. dimerus* in relation with aggressive behavior and reproductive physiology, by measuring steroid levels in plasma and analyzing ovarian histology, during the different phases of the reproductive period.

2. Material and methods

2.1. Animals

C. dimerus, caught in Esteros del Riachuelo (27°35′S; 58°45′W; Corrientes, Argentina), were housed in aquaria under conditions that mimicked their natural habitat [17] at least for one month before starting the experiments. The conditions were (25 ± 1)°C; 14:10 light:dark cycle with full spectrum illumination. A layer of gravel (~4 cm) covered the bottom of the aquaria and natural aquatic plants were placed together with stones that fish use to delimit their territories and lay their eggs. Animals were fed ad libitum every morning with cichlid pellets (Tetra). Appropriate actions were taken to minimize pain or discomfort of the animals, and the experiments were conducted in accordance with international standards on animal welfare as well as being compliant with local and national regulations. All procedures are in compliance with the National Institutes of Health Guide for Care and Use of Laboratory Animals [18]. Animals were kept in communitarian tanks (150 L) in order to allow the establishment of the dominant pair [3]. When the dominant pair was established, it was separated and kept together in a smaller tank (50 L). In total, 40 animals were used for physiological and behavioral experiments.

2.1.1. C. dimerus reproductive cycle

The reproductive cycle of *C. dimerus* females can be divided in reproductive (November–March), and non-reproductive periods (April–October). The different spawning events during the reproductive period were classified as reproductive stages which can be subdivided in four phases, according to the offspring degree of development: female with prespawning activity (FP, day 0), female with eggs (FE, DAF 1), female with hatched larvae (FHL, DAF 3) and female with swimming larvae (FSL, DAF 8).

2.2. Behavioral experiments

In order to study and compare the aggressive interactions of *C. dimerus* females in the 4 different phases of the reproductive stage (FP, FE, FHL and FSL), 12 females previously established with a male ("resident females"), and 12 prespawning females each also paired with a male ("intruder females"), were used. The experiment consisted in the removal of the male from the resident female tank before adding an intruder female for 1 h. All the interactions were digitally recorded (Coolpix 4500, Nikon). The time in which the attacks started was recorded and the aggressive interactions made from resident female to intruder female were described and quantified. The aggressive interactions consisted in bites on lateral body regions, fin, tail and eyes, chasings and mouth holding. Body color pattern and the shape of dorsal fin were also examined before and during the encounter. Females were previously weighed and measured in order to use animals that were equal in size. The experiments were carried out at the same time of the day (between 1:00 and 3:00 pm).

2.3. Physiological parameters

Four females from each reproductive phase were anesthetized by immersion in a 0.1% benzocaine solution. Before sacrifice, blood from caudal veins was sampled for measurement of steroid hormones levels. Dissections of brain, pituitary, ovaries, liver and spleen were made. Blood and tissue sampling were performed at the same time (between 1:00 and 3:00 pm) since hormone levels can vary widely throughout the day.

2.3.1. Gonadosomatic index

Ovaries were weighted and gonadosomatic index was calculated with the following formula: GSI = gonad mass / (total body mass − gonad mass) 100.

2.3.2. Immunohistochemical and morphometrical analysis of GnRH3 neurons

2.3.2.1. Immunohistochemistry. After dissection, brains were fixed in Bouin solution for 24 h at 4 °C, dehydrated and embedded in Paraplast®. Samples were completely sectioned coronally at 12 μm intervals and sections were mounted on charged slides (Fisherbrand Superfrost/Plus, Fisher). Sections were deparaffinized in xylene, rehydrated through a graded ethanol series to phosphate-buffered saline (PBS, pH 7.4), treated for 30 min with PBS containing 5% non-fat dry milk, incubated for 16 h at 4 °C with a 1:2000 dilution of LRH13 monoclonal antibody. This antibody, that recognizes the three GnRH isoforms of this species [19], was generously gifted by Dr K. Wakabayashi (Zoological Institute, University of Tokyo, Japan). The specificity of this antibody was previously demonstrated in this species [19]. Then, slides were washed in PBS, and incubated for 45 min in a biotinilated anti-mouse IgG following manufacturer instructions (Dako, CSA Amplification Kit). In order to amplify the signal, sections were afterwards incubated in streptavidin, tyramide and peroxidase-conjugated streptavidin (Dako) for 30 min each. After three washes in PBS, peroxidase activity was visualized with 0.1% 3.3′-diaminobenzidine (DAB) in TRIS buffer (pH 7.6) and 0.03% H_2O_2. Sections were lightly counterstained with hematoxylin, mounted, examined with a Microphot FX (Nikon) microscope and digitally photographed (Coolpix 4500, Nikon).

2.3.2.2. Morphometrical parameters of GnRH-3 neurons. From the total number of immunoreactive GnRH3 cells in each brain, 10 were randomly selected for measurement of the average nuclear and somatic area (Image Pro-Plus) and optical density of staining (Image Gauge version 3.12, Fuji). To reduce variability within immunohistochemical results, the conditions of the immunohistochemical reactions were controlled and were kept homogeneous on all parameters. As it may be difficult to compare staining intensity among tissues reacted at different times, representatives of females of the four phases were included in each batch of IHC reactions to further control the staining differences. Slides were coded so that the observer was blind to the respective treatments. For the precise location of the GnRH-3 cell nuclei, we relied on the detailed atlases of two other perciform species, *Dicentrarchus labrax* [20] and *Astatotilapia burtoni* [21], and on previous studies of GnRH neurons localization in this species [19,5].

2.3.3. Semiquantification of β-FSH, β-LH pituitary content

In order to semi-quantify pituitary content of gonadotropins (β-FSH, β-LH), an analysis with 15% sodium dodecylsulfate-polyacrylamide gel electrophoresis (SDS-PAGE) followed by Western

blot was performed. The hormones were detected using heterologous antisera against conservative regions of fish gonadotropins: anti-*Fundulus heteroclitus* β-FSH and β-LH [22]. Those antisera were generously gifted by Dr Akio Shimizu (National Research Institute of Fisheries Science, Fisheries Research Agency, Yokohama, Japan). These antisera were specific in the recognition of *C. dimerus* FSH and LH β-subunits as was previously demonstrated [23]. After dissection, each pituitary was homogenized in 100 μl of Tris–HCl buffer 50 mM, pH 7.4, with 1 μl of protease inhibitor cocktail (Sigma-Aldrich). 15 μg of protein with loading buffer (120 mM Tris–HCl pH 6.8, 3% dodecylsulfate, 10% glycerol, 1% β-mercaptoethanol), heated at 100 °C for 5 min and loaded into the gel. After electrophoresis, proteins and molecular markers (SeeBlue Plus2 PreStained Standard; Invitrogen) were transferred to a nitrocellulose membrane (Amersham Biosciences) for 60 min at 4 °C and 75 V. Then, membranes were washed in TBST pH 7.5 and blocked with TBST containing 3% non-fat dry milk overnight. After that, they were incubated for 3 h at room temperature (RT) with both primary antisera and washed in TBST afterwards. Membranes were then incubated with a biotinylated anti-rabbit IgG (Sigma-Aldrich) (1:1000) for 1 h at RT, washed again and finally incubated with a streptavidin complex conjugated to alkaline phosphatase (Sigma-Aldrich) (1: 2000) for 45 min at RT. After washing, the reaction was visualized using an alkaline phosphatase developing kit (BCIP/NBT, Vector Blue, Dako). Finally, membranes were dried; digitalized and optical density semiquantification was performed using Image Gauge Software (version 3.12 Fuji Photo Film) software. In order to avoid a possible loading errors in the SDS-PAGE, pituitary hormone content was semi-quantified by densitometric analysis and normalized to the optical density obtained for α-tubuline.

2.3.4. Plasma cortisol, estradiol and testosterone measurements

Blood was drawn from caudal puncture with heparin-coated syringe, 27 gage × 1/2 in. needle. The time from opening of the tank to completion of blood collection was similar in all fish (less than 4 min). Plasma was separated by centrifuging at 3000 rpm for 15 min and stored at −20 °C until assay. Careful netting and handling was implemented to minimize stress. Cortisol and 17-β estradiol assay was measured by ECLIA (electrochemiluminescence immunoassay), (Cobas analyzer, Roche). Androgens assay was measured by RIA using a heterologous antiserum anti-human testosterone generated in mouse (DSL-4000 ACTIVE-Testosterone Coated-Tube Radioimmunoassay Kit). This antiserum has a 45% cross-reaction with 11-ketotestosterone. This is why results are presented as relative immunoreactivity in arbitrary units. All analyses were carried out according to the manufacturer's instructions and a standard curve was run. Pilot assays using three different dilutions of ten samples (five samples per sex) were run to establish the appropriate working dilution and all samples were assayed in duplicate. The slope of this dilution series was parallel to the standard curve for all kits. Intra- and inter-assay tests were also performed for all used kits. Inter-assay coefficients of variation did not exceed 14.5% in steroid hormones assays.

2.3.5. Ovarian histology

Ovaries were fixed in Bouin solution for 24 h at RT, dehydrated and embedded in Paraplast®. Samples were completely sectioned transversely at 7 μm intervals and mounted on charged slides (Fisherbrand Superfrost/Plus, Fisher, Wash). Sections were deparaffinized in xylene, rehydrated through a graded ethanol series to distilled water. Hematoxylin-eosine and Masson trichromic stainings were performed. Then, sections were dehydrated through a graded ethanol series to xylene and mounted in DPX medium. Sections were examined and photographed with a Microphot FX microscope (Nikon), and digitally photographed (Coolpix 4500, Nikon).

2.4. Statistical analysis

Statistical analysis of behavioral experiments, plasma steroids and organ-somatic indexes was performed using a one way analysis of variance (ANOVA). Statistical analysis pertaining Western blot data was performed using a randomized block ANOVA design. Statistical analysis of data pertaining nuclear and somatic area and optical density or GnRH-3 neurons was performed using a two-way nested ANOVA. All data are presented as mean ± SEM. Statistical significance was established at the $p<0.05$ level. When significant differences were found, ANOVAs where followed by Tukey test. All analyses were performed with the Infostat 2010e Software.

3. Results

3.1. Female aggressive behavior and its comparison among reproductive phases

The number of bites and chases produced by the resident female against the intruder were quantified to compare the aggressive behavior pattern in the different reproductive phases. FP were the most aggressive ones, as they displayed the maximal number of bites to the intruder (315 ± 3.98 bites; $p=0.0016$), when compared to the females in the rest of reproductive phases (FE: 104 ± 49.27 bites; FHL: 148.33 ± 35.25 bites; FSL: 75.33 ± 29.69 bites) (Fig. 1a). FP, FE and FHL did not present significant differences in the number of chases. FSL chased the intruder significantly less than FP resident female (78.33 ± 22.72 vs 14.67 ± 5.03 chases; $p=0.0187$) (Fig. 1b).

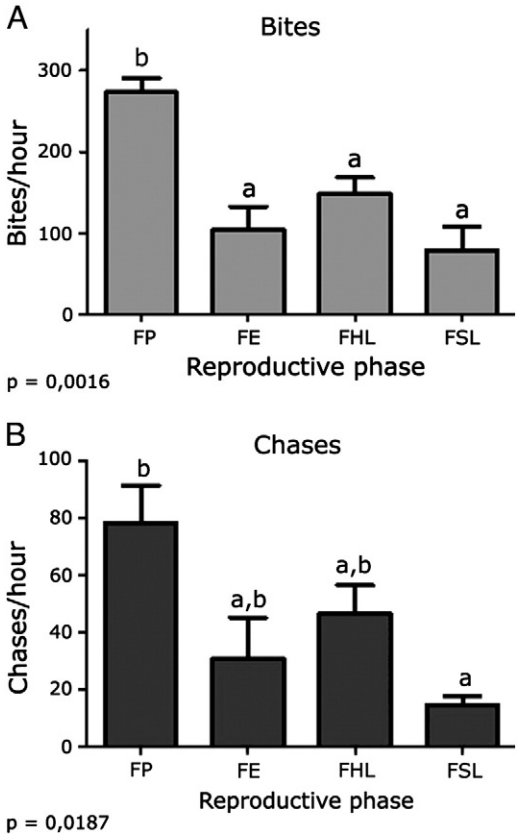

Fig. 1. Number of bites (A) and chases (B) displayed by the resident female against the intruder female during the experiment. Different letters indicate significant differences ($\alpha=0.05$) ($N=12$). Data is presented as mean ± SEM. FP: Females with prespawning activity; FE: Female with eggs; FHL: Females with hatching larvae; FSL: Females with swimming larvae. a. FP displayed more bites to the intruder female than the rest of the females. b. FP chased the intruder female more times than the FSL.

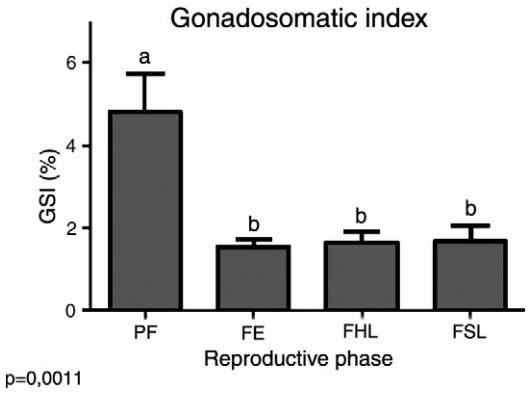

Fig. 2. Gonadosomatic index of females during the different reproductive phases. Different letters indicate significant differences ($\alpha = 0.05$) ($N = 12$). Data is presented as mean ± SEM. FP: Females with prespawning activity; FE: Female with eggs; FHL: Females with hatching larvae; FSL: Females with swimming larvae. a. FP had the highest gonadosomatic index. The other reproductive phases did not present significant differences.

3.2. Gonadosomatic index

Females with prespawning activity showed a higher gonadosomatic index ($4.81 \pm 2.10\%$; $p = 0.0011$) than the females in the rest of the reproductive phases. (FE: $1.55 \pm 0.34\%$; FHL; $1.64 \pm 0.51\%$; FSL: $1.67 \pm 0.93\%$) ($F = 8.77$; $df = 3$) (Fig. 2).

3.3. Morphometry of GnRH-3 neurons among reproductive phases

Neuronal nuclear area, somatic area and relative optical density of staining of ir-GnRH-3 cell bodies were examined. GnRH-3 neurons were mainly located at the *nucleus olfacto retinalis* (NOR) (at the junction of the olfactory bulb and the ventral telencephalon), and they showed a spherical or ovoid nucleus. In general, they appeared organized in clusters, although cellular limits could be distinguished. In some cases they also appeared isolated (Fig. 3a). NOR was the only region analyzed. Even though there are GnRH-3 neurons also in the olfactory bulb and in the ventral telencephalon, in these areas they are less and they are more dispersed.

3.3.1. Nuclear area

Nuclear area was examined only in those ir-GnRH-3 neurons where the nuclear limit was clearly distinguishable. FP ($113.103 \pm 9.62\ \mu m^2$), FE ($99.22 \pm 58.14\ \mu m^2$) and FHL ($103.89 \pm 48.36\ \mu m^2$) showed higher nuclear area than FSL ($82.84 \pm 28.89\ \mu m^2$; $p = 0.0428$). FHL showed higher nuclear area than FE. ($F = 3.42$; $df = 3$) (Fig. 3b).

3.3.2. Somatic area

Somatic area was examined only in those ir-GnRH-3 neurons where the somatic limit was clearly distinguishable. FSL showed a smaller somatic area ($206.71 \pm 74.12\ \mu m^2$) than the females in the rest of reproductive phases ($p = 0.015$). FHL showed a smaller

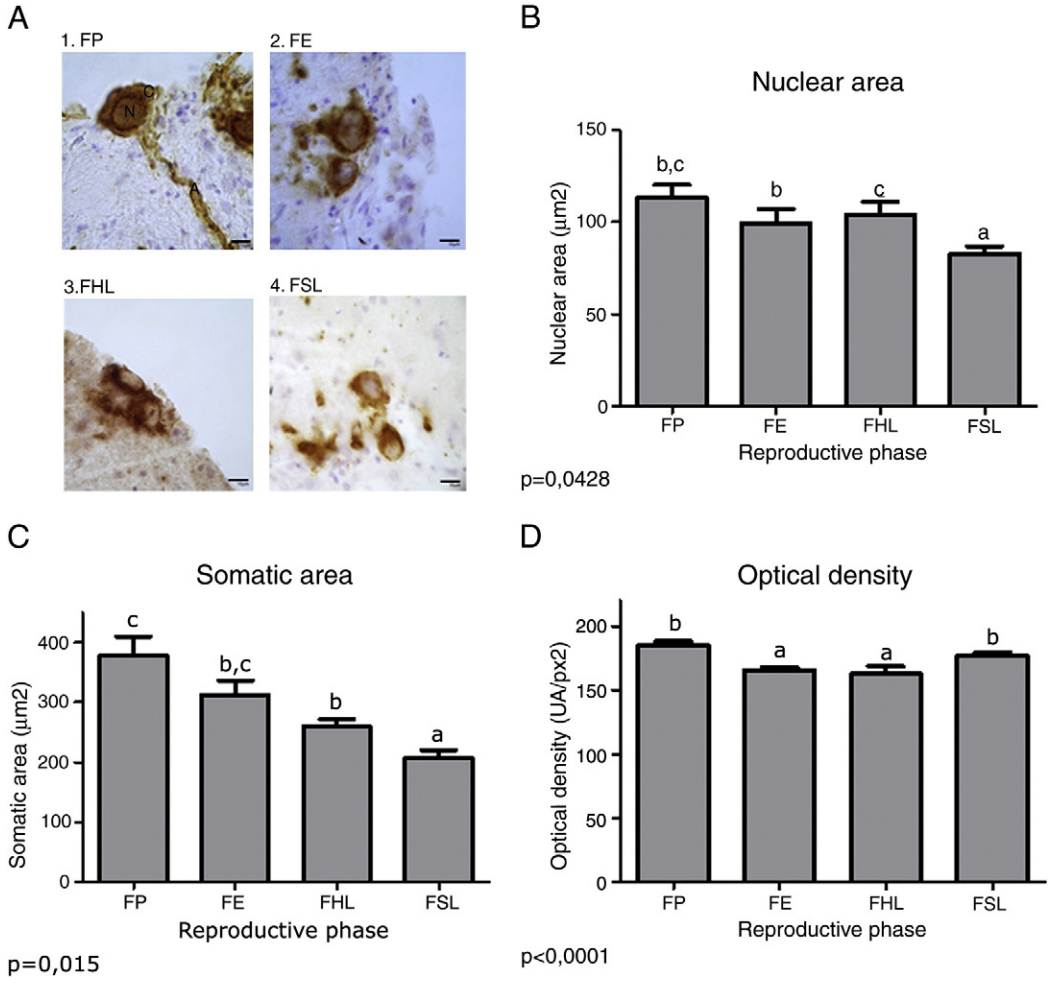

Fig. 3. (A) Light microscope photograph. Sections of *C. dimerus* brain showing ir-GnRH-3 neurons of females during the different reproductive phases. Nuclear area (B), somatic area (C) and optical density (D) of ir-GnRH-3 neurons of females of the reproductive phases. Different letters indicate significant differences ($\alpha = 0.05$) ($N = 12$). Data is presented as mean ± SEM. Bar: 10 µm. FP: Females with prespawning activity; FE: Female with eggs; FHL: Females with hatching larvae; FSL: Females with swimming larvae; N: Nucleus; C: Cytoplasm; A: Axon. b. FSL had the smallest GnRH-3 nuclear area, while FHL had a bigger nuclear area than FE. c. FSL had the smallest GnRH-3 somatic area, and FP had a bigger somatic area than FHL. d. FP and FSL showed higher optical density of staining GnRH-3 neurons than FE and FHL.

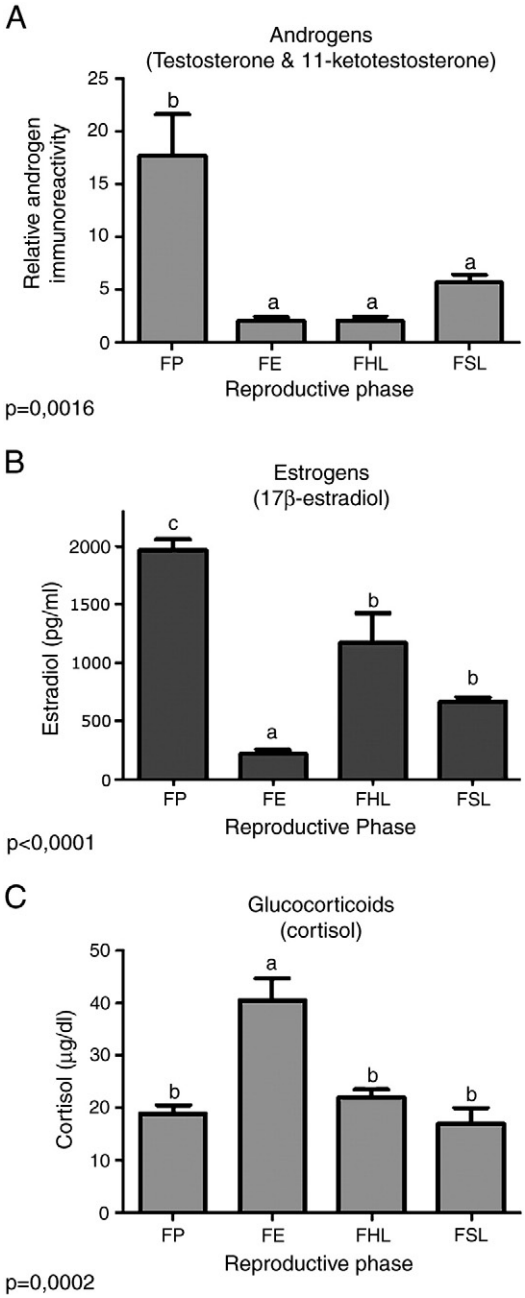

Fig. 4. Androgen (A), estradiol (B) and cortisol (C) levels measured in plasma of females in different reproductive phases. Different letters indicate significant differences ($\alpha = 0.05$) ($N = 12$). Data is presented as mean ± SEM. FP: Females with prespawning activity; FE: Female with eggs; FHL: Females with hatching larvae; FSL: Females with swimming larvae. a. Androgen plasma levels were maximum in FP. The other reproductive phases did not show significant differences. b. Estradiol levels were maximum in FP and minimum in FE. FHL and FSL presented intermediate levels of estradiol in plasma. c. Cortisol plasma levels were maximum in FE. The other reproductive phases did not present significant differences.

somatic area ($259.02 \pm 56.89\ \mu m^2$) than FP ($378.09 \pm 143.04\ \mu m^2$) ($F = 15.29$; $df = 3$) (Fig. 3c).

3.3.3. Relative optical density of staining

Somatic optical density of staining was examined in ir-GnRH-3 neurons with a distinguishable nucleus that did not present overlapping between cell bodies. FP ($159.61 \pm 11.90\ AU/px^2$) and FSL ($142.47 \pm 8.73\ AU/px^2$) showed a higher relative optical density (ROD) ($p < 0.0001$) than FE ($130.26 \pm 13.07\ AU/px^2$) and FHL ($162.39 \pm 13.82\ AU/px^2$) ($F = 66.97$; $df = 3$) (Fig. 3d).

3.4. Semiquantification of β-FSH, β-LH pituitary content during the reproductive phases

The ROD of ir-βFSH and ir-βLH bands (normalized to α-tubulin) was measured. No significant differences were detected in the ROD of β-FSH (19 kDa or 15 kDa bands) (FSH 15 kDa: FP = 218.90 ± 57.66; FE = 171.00 ± 52.18; FHL = 186.10 ± 60.15; FSL = 231.58 ± 9.31; F = 0.32; df = 3; FSH 19 kDa: FP = 137.93 ± 43.27; FE = 97.89 ± 25.71; FHL = 113.17 ± 65.08; FSL = 139.61 ± 36.33; F = 0.20; df = 3) and β-LH 24 kDa (FP = 174.67 ± 42.36; FE = 163.69 ± 53.22; FHL = 200.62 ± 65.36; FSL = 222.39 ± 21.33; F = 0.30; df = 3) in females among the studied reproductive phases.

3.5. Plasma steroid profiles along female reproductive phases

3.5.1. Androgens

FP showed higher androgen levels — expressed in relative androgen immunoreactivity, arbitrary units (au) (17.73 ± 6.74 au; $p = 0.0016$) than females on the rest of reproductive phases (FE: 2.03 ± 0.67 au; FHL: 2.05 ± 0.78 au; FSL: 5.70 ± 1.35 au) ($F = 13.74$; $gl = 3$) (Fig. 4a).

3.5.2. 17-β Estradiol

FP showed the highest estradiol levels (1966.67 ± 152.75 pg/ml; $p < 0.0001$) between the phases studied, while FE showed the lowest one (214.0 ± 69.20 pg/ml). FHL and FSL showed intermediate levels of estradiol in plasma (FHL: 920.0 ± 208.81 pg/ml; FSL: 665.0 ± 83.47 pg/ml) ($F = 92.04$; $df = 3$) (Fig. 4b).

3.5.3. Cortisol

FE showed higher levels of cortisol in plasma (40.50 ± 8.23 μg/ml; $p = 0.0002$) than the other female phases (PF: 18.85 ± 3.42 μg/ml; FQL: 22.0 ± 2.94 μg/ml; FSL: 17.0 ± 6.06 μg/ml) ($F = 14.99$; $df = 3$) (Fig. 4c).

3.6. Ovarian histology through the reproductive phases

Using histological techniques, ovarian histology was characterized along the different reproductive phases. Among these, marked differences on the abundance and preponderance of the different stages of the oogenesis in the ovarian lamellae were observed. *Cichlasoma dimerus* possesses saccular paired ovaries – about 3 cm total length – connected directly to the urogenital papilla. Ovaries are located into the body cavity, supported by a mesovarium and surrounded by an ovarian capsule (Fig. 5a, b). As this species has a type of asychronous ovary, other stages of the oogenesis were simultaneously easily seen within the ovary, such as: a) *primary growth oocytes*: previtellogenic, the smallest cell type, with central nucleus and peripheric nucleoli, basophilic cytoplasm, surrounded by a layer of flat follicular cells, and b) *secondary growth oocytes*: (b.1) early vitellogenic oocytes with peripheral cortical granules, some lipid droplets and slightly basophilic cytoplasm, or (b.2) vitellogenic oocytes, with dense yolk granules and lipid droplets, acidophilic cytoplasm, surrounded for a layer of cubic follicular cells (Fig. 5b). *FP* presented ovaries with a preponderance of mature oocytes; showing advanced vitellogenic stages, acidophilus cytoplasm, and peripheral nucleus (Fig. 5c). *FE* presented ovaries with a preponderance of post ovulatory follicles (POF), which are hypertrophied follicular and tecal cells surrounding the space previously occupied by the ovulated oocyte. Also, primary and secondary growth oocytes were observed in these ovaries (Fig. 5d). *FHL* presented ovaries with a preponderance of atresic follicles which represent the degradation of the mature oocytes that were

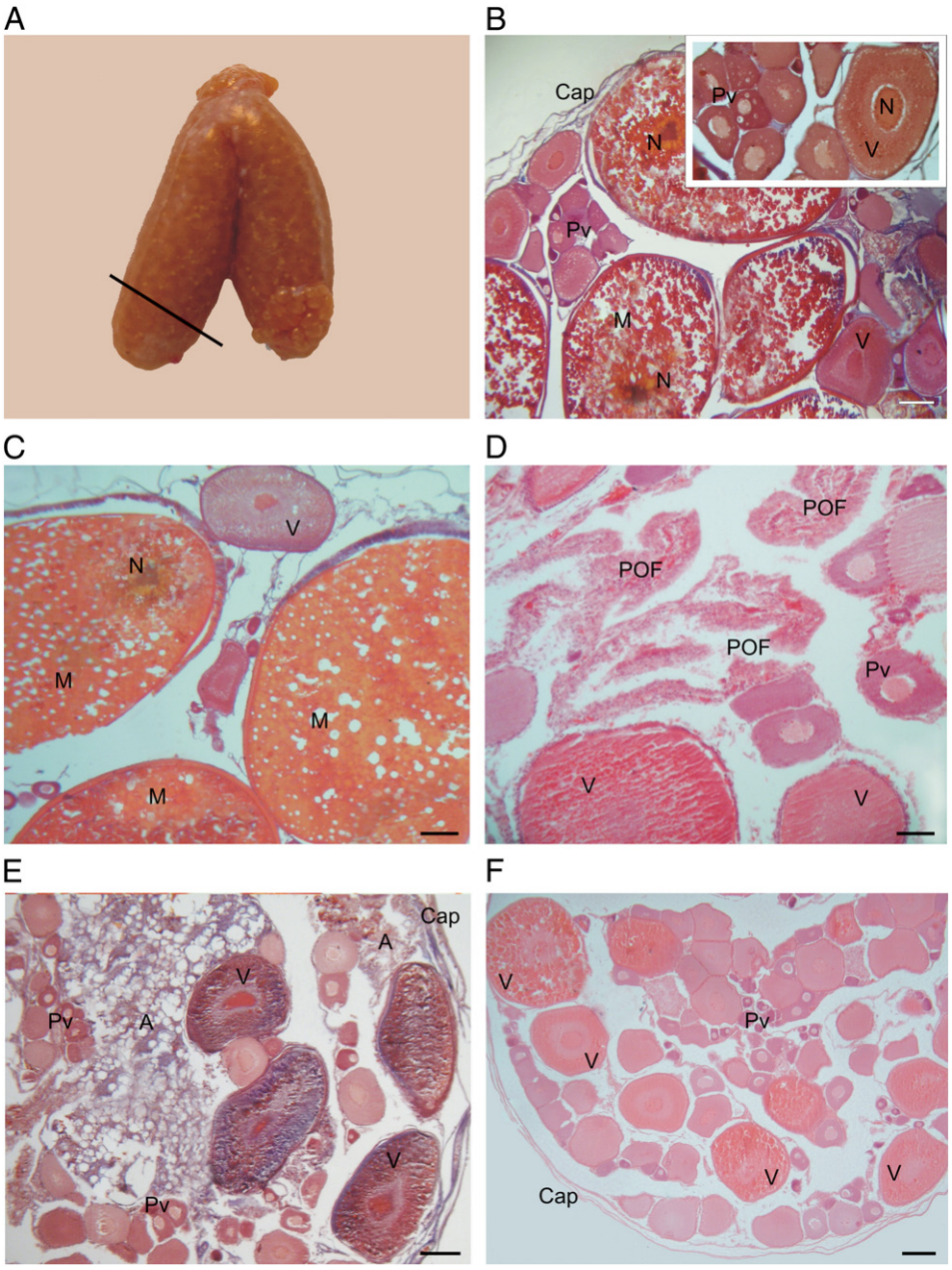

Fig. 5. A. Macroscopic aspect of *Cichlasoma dimerus* ovaries (3 cm total length). The black line indicates the position of the transverse sections shown from b to f. B. Photomicrograph of a transverse section of a FP ovary at a low magnification in order to appreciate its general aspect. Inset. Different stages of early oogenesis (primary and secondary growth oocytes). C. Detail of FP ovary, with a preponderance of mature oocytes. D. Detail of FE ovary, with a preponderance of POF. E. Detail of FHL ovary, with a preponderance of atresic bodies. F. Detail of FSL ovary, with preponderance of oocytes in secondary growth. (N = 12). A: Atresic bodies; Cap: ovarian capsule; M: Mature follicles; N: oocyte nucleus; POF: Post ovulatory follicle; PV: Previtellogenic oocyte (primary growth); V: Vitellogenic oocyte (secondary growth). Bar: 10 μm.

not ovulated. These ovaries also presented oocytes in primary and secondary growth (Fig. 5e).

FSL presented ovaries with preponderance of primary and early secondary growth, indicating an important growth activity after the mature oocyte were spawn (Fig. 5f).

4. Discussion

Reproductive behavior related either with mating system or with offspring care is of particular importance for a successful reproduction [24]. *C. dimerus* present biparental care behaviors such as fanning and cleaning the eggs, transferring of newly hatched larvae to a previously dug pit, and keeping all larvae together when they start swimming freely. Related with this, body color patterns are displayed by both parents, together with aggressive behaviors towards non-dominant individuals.

In most of the studies, aggressive behavior is studied in males of social species, when hierarchy establishes, defending a territory or at mating time. In this work, we observed that females of *C. dimerus* were very aggressive during the reproductive period, with different degrees along the reproductive phases. Females with prespawning activity were the most aggressive ones. This was evidenced by the highest number of biting and chases towards the intruder female displayed during the experiment. This result could be explained by the fact that these females didn't have to display parental care yet, so that they had all their energy involved in defending their territory.

In the wild, this species is found in groups, within which a social hierarchy is established between the dominant and non-dominant members of the group, and also between non-dominant individuals.

The experimental design used may replicate a natural situation, where the dominant male is captured by a predator, and the female was left alone either with prespawning activity or with the fry, with other individuals that will be able to access to her territory.

Several hormones are involved in the modulation of specific behavioral patterns and this occurs by acting on neural circuits that influence a given behavior. In teleosts, like in the rest of the vertebrates, growth and reproduction are strongly regulated, mainly by the hypothalamus-pituitary-gonadal axis. The gonadotropin-releasing hormone (GnRH) plays a major role in the regulation of this axis. GnRH system in several teleost fish is represented by three different populations (GnRH-1, GnRH-2 y GnRH-3), with different functions. It was suggested, in numerous studies, that GnRH-3 is a potent neuromodulator of the reproductive behavior in some teleost species. In particular, it has been related with the behavioral response to photoperiod in *C. dimerus* [25], and with the nest building and aggressive behavior in the tilapia *O. niloticus* [6]. GnRH-3 neurons have been mostly studied in male fish. In this work we studied morphometrical parameters of these neurons in females of *C. dimerus*, and we found differences among phases of the reproductive stage. In concordance with the pattern observed on the aggressive behavior, nuclear and somatic area, and optical density of GnRH-3 neurons were higher in females with prespawning activity than in females with eggs or larvae. The biggest nuclear area may be reflecting an increase in protein synthesis [26]. FHL presented a bigger nuclear area than FE, which may imply an increase in the synthesis activity after larvae hatching. Females with free-swimming larvae presented the smallest nuclear area, and this may be indicating a lowest synthesis of protein that correlates with the lowest aggressive behavior observed in this stage. The biggest somatic area together with a major optical density may be indicating protein accumulation in the cytoplasm of the cell. Females with prespawning activity presented both parameters in their maximal expression within the phases studied. Somatic area of GnRH-3 neurons got reduced along the studied reproductive phases. A decrease is observed first in FHL. FSL presented the minimum somatic area. This pattern may imply that along the reproductive stage, females presented less amount of the GnRH-3 peptide in the cytoplasm of these neurons. On the other hand, optical density was maximal in females with prespawning activity, but then it decreased abruptly in females with eggs and remained low in females with hatching larvae. This would reflect a big release of the peptide during spawning. After the increase of the synthesis process in FHL, FSL showed a new increase in GnRH-3 optical density of staining, probably reflecting accumulation of the peptide in the cytoplasm for a new prespawing stage [27].

GnRH-1 population projects their axons to the pituitary, where it induces the synthesis and release of gonadotropins LH and FSH. The relative optical density (R.O.D.) of FSH and LH bands did not show significant differences between females of different reproductive phases. It is possible that the fluctuations of these hormones are not marked, and could not be detected by a semiquantitative technique such as Western blot, as this is not the most accurate methodology. The two bands observed in the FSH membrane are specific, as they disappear in the preadsortion tests, but it is still unclear if they correspond to different isoforms, different degree of glycosilation or a possible partial degradation [23].

Differences observed in gonadal histology of females of different reproductive phases are reflected in their GSIs. Mature oocytes are bigger and occupy more volume within the ovary, and this is in direct relation with the fact that females with prespawning activity had the highest GSI. Primary and secondary growth of the oocytes do not involve great changes of size, and this is reflected in the fact that GSI of FE, FHL and FSL did not differ significantly. Further studies beyond day 8 postspawning are needed to determine how long it takes for this species to start a new spawning stage.

17-β-estradiol plasma levels were highest in females with prespawning activity. This result is similar of what occurs in mammals that present a preovulatory increase in plasma estradiol. The major androgens synthesized in fish are testosterone (T) and 11-ketotestosterone (11-KT); T can be converted into 11-KT. Both are released to circulation, although T is mainly converted to estradiol by aromatase. 11-KT is not aromatizable, so it is released to blood like that. However, at least in female mammals, androgen synthesis occurs both in the ovaries and in the adrenal gland [28]. Little is known about androgen synthesis in female fish, although it was established that the "interrenal gland" of fish synthesizes T that is released to circulation [29]. Results obtained in this work showed that females with prespawning activity presented the highest concentration of plasma androgens. In the rest of the phases androgens remained low. It is important to highlight the 45% of cross-reactivity of T and 11-KT with the antibody used, in order to interpret the obtained results with caution. For example, the obtained measurement for FP can be indeed 17.7 ng/ml of T or 59.3 ng/ml of 11-KT, or a mix of both. That is the reason why we decided to express the results in relative androgen immunoreactivity, arbitrary units. It was also suggested that androgens increased the aggressive behavior both in males and females [15,30,16]. Both plasma androgen levels pattern observed in *C. dimerus* females and aggressive behavior exhibited higher levels during the same reproductive stage. Hormones may increase or decrease the probability of occurrence of specific behavior [31]. Therefore, androgens might be mediating these aggressive behaviors in females, interacting with specific receptors at the central level and with specific neural circuits that underlie that behavioral pattern. In concordance with these results, in other cichlid fish, *N. pulcher* it was demonstrated that dominant females, few hours after the confrontation with other female to establish hierarchy, showed increased levels of testosterone but not of 11-KT [14]. These results suggested that changes in physiology and behavior of dominant fishes after a confrontation that involves aggressive interaction are mediated by androgens [14].

The major product of "interrenal" steroideogenesis in fish is cortisol, which increases dramatically its plasma levels during stress [32]. In this work we found that females with eggs presented the highest concentration of plasma cortisol. It is possible that spawning is a novel and stressing event during the reproductive stage, which increases cortisol levels in plasma. Non dominant individuals, both males and females of this species, present also higher levels of plasma cortisol than dominant ones [3]. On the other hand, dominance is associated with higher levels of aggressiveness. Therefore, our results showed that females with higher levels of cortisol had low levels of aggressiveness, at least in FE. There are some evidences showing that corticosteroids suppress aggressiveness by increasing levels of serotonin [33].

On the other hand, cortisol was found to regulate calcium uptake in fish, acting as an hypercalcemic hormone [34]. And it was demonstrated that in salmon, calcium decreases in plasma during the spawning [35]. Our results may suggest that cortisol in females of *C. dimerus* plasma increases after spawning to upregulate the intake of calcium in plasma that decreased after spawning.

In summary, at a behavioral and physiological level, we characterized *C. dimerus* females during four phases of the reproductive stage. We found that females with prespawning activity were the most aggressive ones having GnRH-3 neurons with bigger nuclear and somatic area and higher optical density. They also presented the highest levels of plasma androgen and estradiol levels and the maximum gonadosomatic index. From these results we can propose a basic regulation model of the HPG axis. GnRH-1 and GnRH-3 are upregulated by the peptide kisspeptin [36–39]. GnRH-1 stimulates LH and FSH release in the pituitary, which, in turn, stimulate oogenesis and estradiol, 11-KT and T synthesis in the female ovary. On the other hand, kisspeptins also stimulates GnRH-3 release [37] in the pituitary, specifically in the pars distalis and pars intermedia [40]. In pars distalis, GnRH-3 might induce ACTH release. This hormone, besides stimulate cortisol synthesis in interrenal gland, also

stimulates interrenal androgens like T [41]. T could be acting centrally stimulating neuronal circuits involved in aggressive behaviors. Following a temporal patterns it could be established that an increase in kisspeptin neuron's activity during prespawning would stimulate GnRH-1 release, on one hand, that regulate FSH and LH release in pituitary. These hormones induce oogenesis and, in FP would cause the estradiol preovulatory peak. On the other hand, kisspeptins induce an increase in GnRH-3 release, evidenced by a decrease in soma size and relative optical density of these neurons in FE. During the spawn, GnRH-3 would stimulate directly T synthesis, which is maximal in FP. In the central nervous system, T might mediate aggressive behavior, with is also maximal in this reproductive phase. In FE cortisol might increase as a stress reaction in response to an acute release of adrenalin or noradrenalin. Cortisol might inhibit ACTH release by negative feedback, and so, T release would decrease in the next phase.

In future experiments GnRH-3 and steroid levels in females from the four reproductive phases immediately after the aggressive confrontation will be analyzed.

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