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The interrenal gland in males of the cichlid fish *Cichlasoma dimerus*: Relationship with stress and the establishment of social hierarchies

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ABSTRACT

In teleosts, cortisol is the primary glucocorticoid secreted by the steroidogenic cells of the interrenal gland and an increase in its plasma concentration is a frequent indicator of stress. Cortisol has been postulated as an endogenous mediator involved in the regulation of reproduction and aggression related to social dynamics. The cichlid fish *Cichlasoma dimerus*, is a monogamous species that exhibits complex social hierarchies; males appear in one of two basic alternative phenotypes: non-territorial and territorial males. In this work, we postulated as a general hypothesis that the morphometry of the interrenal gland cells and the plasma levels of cortisol and 11-ketotestosterone (11-KT) are related to the social rank in adult males of *C. dimerus*. First, the location and distribution of the interrenal gland with respect to its context – the kidney – was studied. Plasma levels of cortisol and 11-KT in territorial and non-territorial males were established by ELISA. Finally, a morphometric analysis of steroidogenic and chromaffin cells of the interrenal gland was performed. Results showed that the interrenal gland was exclusively located in the posterior portion of the cephalic kidney. Non-territorial males presented a greater nuclear area of their steroidogenic cells. Additionally, plasma cortisol and 11-KT levels were lower and higher, respectively, in territorial males. Finally, plasma cortisol levels positively correlated with the nuclear area of interrenal steroidogenic cells. Thus, the interrenal gland, by means of one of its products, cortisol, may be fulfilling an important role in the establishment of social hierarchies and their stability.

1. Introduction

A wide range of stimuli challenge animals during their lives. These stimuli – namely stressors – are addressed by generating physiological responses to those circumstances. The way in which an event affects an organism, both in natural and captive conditions, depends on its nature, duration, intensity and frequency and the inherited or acquired capacity to cope with it (Barton, 2002).

In teleost fish, social hierarchies are a major source of psychological and physical stress. Many studies have evaluated the relationship between social context and the neuroendocrine axes involved in the stress response in teleosts (Alonso et al., 2012; DiBattista et al., 2005; Earley et al., 2006; Fox et al., 1997; Gilmour et al., 2005; Sørensen et al., 2011, 2012). In general, dominant individuals hold the highest position within the social hierarchy, taking possession of valuable resources related to food, reproduction or defense (Chichinadze and Chichinadze, 2008; Sloman et al., 2000), whereas subordinate individuals are excluded from all or some of them; aggression is, in most of the cases, an essential prerequisite for the establishment of social hierarchies (Desjardins et al., 2012; Parikh et al., 2006; Sloman et al., 2000).

There is an increasing interest in the role played by cortisol, the main corticosteroid produced by teleost fish (Barton and Iwama, 1991; Mommsen et al., 1999), in terms of the physiological and behavioral changes associated with low social rank (Gilmour et al., 2005). Subordinate males usually exhibit higher plasma cortisol levels than dominant ones (Alonso et al., 2011; Øverli et al., 1999; Pottinger and Pickering, 1992; Winberg and Lepage, 1998). If these levels are elevated for a prolonged period of time, subordinate individuals might be subjected to chronic stress, which has a detrimental effect on the animal's physiological state (Gilmour et al., 2005). For example, in terms of its effects at the gonadal and reproductive level, a chronic increase in plasma cortisol can inhibit the production of 11-ketotestosterone (11-KT), the most potent androgen in teleost males, by competitive inhibition of

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two enzymes involved in the conversion of testosterone (T) to 11-KT (Consten et al., 2001).

In teleosts, cortisol is synthesized and secreted by steroidogenic cells (Idler and Truscott, 1972), which in addition to chromaffin cells constitute the adrenal gland (Hanke and Kloas, 1995), also known as interrenal gland (Grassi Milano et al., 1997). These cells, homologous to those of tetrapod vertebrates, are arranged in groups or chords, in contact with the main veins and their tributaries present in the kidney, primarily in the anterior or cephalic portion (Grassi Milano et al., 1997). Very few studies have evaluated the relation between the morphology of the interrenal gland cells and male's social rank; in particular, it was found that steroidogenic cells exhibited greater synthetic activity in subordinate male rainbow trout (Oncorhynchus mykiss) when compared to dominant ones (Noakes and Leatherland, 1977). Similar results were obtained in subordinate male green swordtails (*Xiphophorus helleri*) (Scott and Currie, 1980).

The South American cichlid fish, *Cichlasoma dimerus* (Heckel, 1840), inhabits the Parana and Paraguay Rivers' basins and has recently been used as a laboratory model to study teleost reproduction, neuroendocrinology and behavior (Alonso et al., 2011, 2012; Fiszbein et al., 2010; Pandolfi et al., 2009; Ramallo et al., 2012; Tubert et al., 2012). In this species, under laboratory conditions, the largest male usually emerges as the dominant and reproductively active male of the group. All non-dominant males maintain a linear hierarchy, established and sustained by aggressive and submissive displays that have already been identified and characterized (Alonso et al., 2011). Subordinate and dominant males differ in many physiological and behavioral attributes; e.g., dominant males have lower plasma cortisol levels (Alonso et al., 2012). Thus, *C. dimerus* is an appropriate model in which to evaluate the relationship between social context and morphological, physiological and behavioral traits.

We hypothesized that there would be a relationship between the social rank of adult males of *C. dimerus* and the morphometry of the interrenal gland cells, as well as with plasma levels of cortisol and 11-KT. Therefore, the first aim of this study was to describe the anatomy and histology of the interrenal gland and its spatial context – the kidney. Secondly, we analyzed whether there were any differences between dominant males and those of lowest social rank in terms of: (a) their plasma levels of cortisol and 11-KT and (b) the morphometry of the interrenal cells.

2. Materials and methods

2.1. Animals

Adult specimens of *C. dimerus* used in this study were captured in Esteros del Riachuelo (27°25′S, 58°15′W) (Corrientes, Argentina). Animals were housed in 350 L aquaria of under conditions mimicking their natural habitat (Alonso et al., 2011; Casciotta et al., 2002) for at least one month before starting the experiments: photoperiod (14:10 light:dark) and temperature (25° ± 2 °C). Afterwards, they were transferred in groups of 8 individuals (4 males and 4 females) to 150 L community aquaria (same photoperiod and temperature) to allow the establishment of social hierarchies. Animals were fed to satiation every morning with commercial cichlid pellets (Tetra®).

Appropriate actions were taken to minimize pain or discomfort of the fish, and the experiments were conducted in accordance with international standards on animal welfare, as well as being compliant with local and national regulations (Comité Nacionál de Ética en la Ciencia y la Tecnología of Argentina). All procedures were in compliance with the Guide for Care and Use of Laboratory Animals (eight ed. 2011, National Academy Press, Washington, p. 220.).

2.2. Description of the interrenal gland and its context

2.2.1. Histological study

For the description of the interrenal gland and its context – the kidney – we used adult males and juveniles. Juveniles were taken from clutches of breeding pairs formed in community aquaria. Seven days after hatching, larvae were transferred to 15 L tanks and held there until the juvenile stage (45 days post-hatch) (Meijide et al., 1840). At this stage, skeletal cartilage calcification has not yet begun but the external and internal morphology resembles that of the adult. Feeding of the juveniles up to the juvenile stage was carried out with larvae of *Artemia* sp. and crushed flakes.

Adult males were anesthetized with 0.1% benzocaine and killed by decapitation at the level of the preoperculum so that the whole kidney remained in the trunk. An abdominal incision was made from the urogenital pore to the anterior end of the trunk to access the abdominal cavity. The kidney was excised (in some occasions, the fixative was poured on the kidney prior to its excision to reduce its rupture during dissection), fixed by immersion in Bouin's solution for 24 h at room temperature, dehydrated (through a descending series of alcohols: 100%, 96%, 90% and 70%), clarified with xylene and embedded in paraplast. The whole kidney was fixed because it was not known where the interrenal gland was located. Samples were then sectioned at 7 μm intervals and mounted on gelatin-coated slides. Afterwards, these sections were stained with Masson trichrome (hematoxylin 8′; acid fucsin-xylidine ponceau 4′; phosphomolybdic acid 5′ and aniline blue 5′) or Periodic acid-Schiff (PAS) (Schiff reagent 7′; hematoxylin 6′), examined with a Microphot FX (Nikon) microscope and digitally photographed (Coolpix 4500, Nikon).

Juveniles were anesthetized with 0.1% benzocaine and the whole specimens were fixed by immersion in Bouin's solution for 24 h at ambient temperature. Subsequently, they were processed in the same way as in adults.

2.2.2. Immunohistochemical study

Some of the samples sectioned at 7 μm were deparaffinized in xylene, rehydrated through a graded ethanol series to phosphate-buffered saline (PBS, pH 7.4) and treated for 30 min with PBS containing 5% non-fat dry milk. Then, sections were incubated for 48 h at 4 °C in a moist chamber with 1:1000 primary antibody (rabbit anti-tyrosine hydroxylase; Millipore, Berdford, MA; catalog No. AB152). This antiserum has been previously well characterized and used successfully to identify dopaminergic cells in the cichlid fish *Astatotilapia burtoni* (O'Connell et al., 2001). To avoid false positives, the technique was assessed by the lack of reaction when the antiserum was substituted with PBS. Sections were washed in PBS and incubated for 60 min with a biotinylated anti-rabbit IgG (Sigma®) diluted 1:600. Amplification of the signal was achieved by incubating the sections with peroxidase-conjugated streptavidin (STRP-HRP) (Dako) diluted 1:600 for 60 min in the dark and 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 haematoxylin, cover-slipped, examined with a Microphot FX (Nikon) microscope and digitally photographed (Coolpix 4500, Nikon). *C. dimerus*' brain sections were used as positive controls.

2.3. Relationship among social rank, plasma 11-ketotestosterone and cortisol levels and interrenal gland cell morphometry in adult male C. dimerus

To study whether a given social rank was related to the morphology of the interrenal gland cells and plasma levels of cortisol and 11-KT in *C. dimerus'* adult males, the territorial, dominant male (pre-spawning stage) (T) and the non-territorial, subordinate male of lowest social rank (NT) from community aquaria (150 L)

containing 8 animals were used. The experiments were carried out between January and March 2012, always at the same time of day in order to minimize the possible effects of circadian variations in hormonal concentrations.

The establishment of the social hierarchies in the community aquaria took 4–7 days, based on their characterization by means of a dominance matrix (Lenher, 1996). Briefly, a dominance index was calculated for each individual based on aggressive and submissive displays exhibited by them while being filmed for one hour. These displays have already been identified, characterized and quantified for *C. dimerus* in different experimental designs (Alonso et al., 2011, 2012; Ramallo et al., 2012). T males were those who presented an active territorial defense through aggressive interactions and exhibited no submissive displays, while the opposite was shown by the NT males.

Once T ($n = 10$; mass: 40.22 ± 3.53 g; total length: 11.87 ± 1 cm; standard length: 9.08 ± 0.83 cm) and NT ($n = 10$; mass: 19.43 ± 1.61 g: total length: 9.39 ± 0.57 cm; standard length: 7.19 ± 0.40 cm) males were identified, they were anesthetized, total and standard length and body mass were measured, and blood samples were taken (see Section 2.3.1. for details) to determine plasma levels of cortisol and 11-KT. Animals were anesthetized with 0.1% benzocaine and euthanized by decapitation and gonads, liver and spleen were weighed to determine the respective organosomatic indexes (OI) calculated as OI = organ mass/(total body mass − organ mass). Finally, and once the location of the interrenal gland was established, the kidney was excised and processed as explained in the Section 2.2.1 to evaluate the morphometry of chromaffin and steroidogenic cells (details in Section 2.3.2.).

2.3.1. Hormonal assay

To minimize possible effects of circadian variations in hormonal concentrations, samples were collected between 12:30 and 14:30. Following subject identification, blood samples (100–300 μL) were collected immediately after netting (less than 4 min) by caudal vein puncture in to heparinized tubes. It has been reported in the cichlid fish *A. burtoni* that after 4 min, plasma cortisol level rises due to manipulation (Fox et al., 1997). Plasma was separated by centrifuging the samples at 3000 rpm for 15 min and stored at −20 °C until assayed.

Plasma 11-KT and cortisol levels were measured using commercial ELISA kits (Cayman Chemical Company, MI, USA for 11-KT and Diagnostics Biochem Canada Inc. for cortisol). Analyses were carried out strictly following the manufacturer's instructions and a standard curve was run for each ELISA plate. Pilot assays using three different dilutions of five samples were carried out in order to establish the appropriate working dilution (plasma diluted 6× for 11-KT and undiluted for cortisol) and all samples were assayed in duplicate. The detection limit of the assay was 4 ng/ml for cortisol and 1.3 pg/ml for 11-KT. Plasma validation was assessed computing intra-assay and inter-assay coefficients of variation (CVs); these were 11.3% and 10.2% for cortisol and 11.1 % and 5.4% for 11-KT, respectively. Parallelism of the dilution curves (4 different dilutions) of the plasma samples had a correlation coefficient of 0.9633 for cortisol and 0.9919 for 11KT after log transformation (Mills et al., 2010).

2.3.2. Morphometric analysis of chromaffin and steroidogenic cells

Nuclear profile area was computed from digital images of interrenal cells at 600× with the software Image Pro Plus (Media Cybernetics) which was previously calibrated with a stage micrometer. All nuclear areas (μm^2) were measured as the cross-sectional area from a group of chromaffin and steroidogenic cells by tracing the cells nucleus profile with a digitizing pen. Fifteen randomly chosen steroidogenic and chromaffin cells with a clear nucleus were measured for each animal. The nuclear area has previously been used as an indicator of cellular activity in fish interrenal cells (Metcalfe, 1998).

2.3.3. Statistical methods

All statistical analyses were performed using Infostat 2010 software (FCA, Universidad Nacional de Córdoba, Argentina); all data fulfilled the criteria for parametric statistics. Organosomatic indexes (OI) were compared by one-way ANOVA, with a threshold for statistical significance corrected by sequential Bonferroni. Statistical analysis of cortisol and 11-KT plasma levels was performed by one-way ANOVA; statistical significance was adjusted using sequential Bonferroni. Measures of the nuclear area of the interrenal gland cells were compared by one-way ANOVA with two nested factors, establishing a threshold for statistical significance corrected by sequential Bonferroni. We investigated whether there was any correlation between plasma levels of cortisol or 11-KT and the nuclear profile area of steroidogenic cells. For this, the nuclear areas of the 15 randomly chosen steroidogenic cells from the previous section were averaged and correlated with plasma levels of cortisol or 11-KT for each animal. Because no correlation with plasma 11-KT from T males was observed, data from T and NT males for this hormone were correlated separately with the nuclear area of steroidogenic cells. All correlations were assessed by calculating the Pearson coefficient, setting the statistical significance at $p < 0.05$. Data are presented as mean ± SEM.

3. Results

The anatomical, histological and immunohistochemical results obtained in the present study were clearly consistent between all analyzed specimens and can be considered as representative of the species.

3.1. Anatomical and histological description of the kidney and the interrenal gland

3.1.1. Kidney

In adult males of *C. dimerus*, the kidney presents as a "Y"-shaped organ and is red and dark brown in color (Fig. 1) located in a retroperitoneal position (as in all vertebrates). Although the kidney constitutes a continuum, it can be divided into two regions with singular anatomical, histological and functional characteristics. The posterior or trunk kidney (TK) is in intimate contact with the ventral surface of the vertebral column, strongly attached to the vertebrae, ribs and their associated musculature; in some individuals it expands through the ventral surface of the dorsal region of the ribs (Fig. 1). The anterior or cephalic kidney (CK) is bilateral and located at the margins of the first vertebra (Fig. 1). Both portions of the kidney – cephalic and trunk – are connected by a thin portion of tissue whose histological characteristics highlight the gradualism with which the differences between the two portions occur. Macroscopically, black spots can be seen on the surface of the whole kidney (Fig. 1), corresponding to melano-macrophage centers.

Although some differences in histology exist between adult and juvenile specimens, the external and internal morphology of juveniles resembles that of adults. Thereby, their incorporation into this study allowed us to clarify the relationship between the kidney and the axial skeleton, since the size of the juveniles permits the observation of a large number of structures in a single slide, in addition to the fact that skeletal cartilage calcification has not yet begun. In juveniles, the CK is located just caudal to the skull and anterior to the swim bladder; in its dorsal portion it continues with the TK (Fig. 2a). The TK arises from the junction of the bilat-

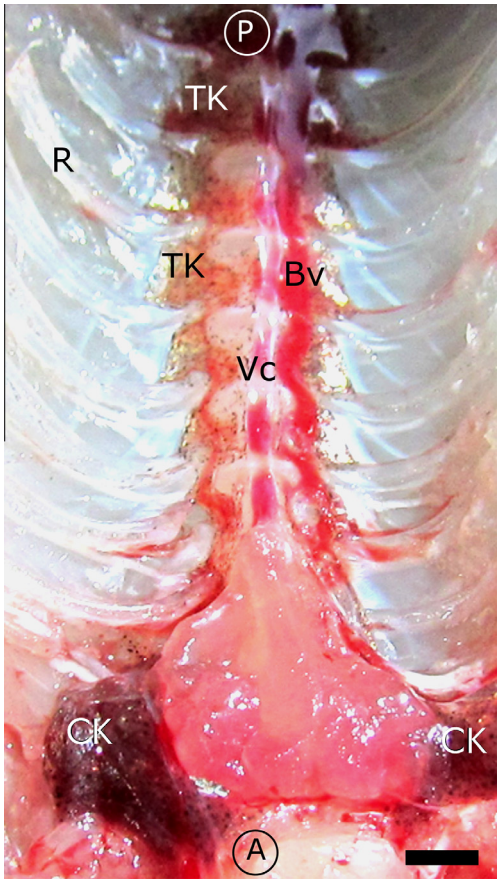

Fig. 1. Photograph of the kidney of an adult male of *Cichlasoma dimerus*. The trunk kidney (TK) is in intimate contact with the ventral surface of the vertebral column (Vc) and the ribs (R), and is parallel to the main blood vessels (Bv) present in this area. The cephalic kidney (CK) is bilateral; black dots over the surface of both portions of the kidney are melano-macrophage centers. A: anterior; P: posterior. *Scale bar:* 5 mm.

eral CK (Fig. 2b), and is in intimate contact with the dorsal musculature and skeletal elements associated with the vertebral column (Fig. 2c).

3.1.1.1. Cephalic kidney. In the CK of adult males, hematopoietic cells form a dense parenchyma and are predominant over any other cell type, showing a great variability in terms of their size and nuclear and cellular shape (Fig. 3a and c). Melano-macrophage centers are nodular structures composed primarily of macrophages surrounded by the parenchyma of hematopoietic cells (Fig. 3a). Employing histochemical techniques, such as PAS, the presence of neutral glycoconjugates associated with macrophages was confirmed (Fig. 3d). In the posterior portion of the CK, blood vessels are abundant and large; these vessels are mostly veins, possibly tributaries of the posterior cardinal vein, which runs in parallel to the vertebral column and branches in the CK. It is important to note that only within this portion of the CK – the posterior one – are interrenal gland components found (Fig. 3b). Other structures present in this portion of the kidney are non-myelinated nerve fibers (Fig. 3b) and nerve ganglia – neure associated with glial cells – (Fig. 3e). The entire CK is surrounded by a thin capsule of densely packed connective tissue (Fig. 3c).

3.1.1.2. Trunk kidney. The parenchyma of adult males is primarily composed of different portions of the nephron (renal corpuscles and renal tubules) and collecting ducts, in addition to large blood

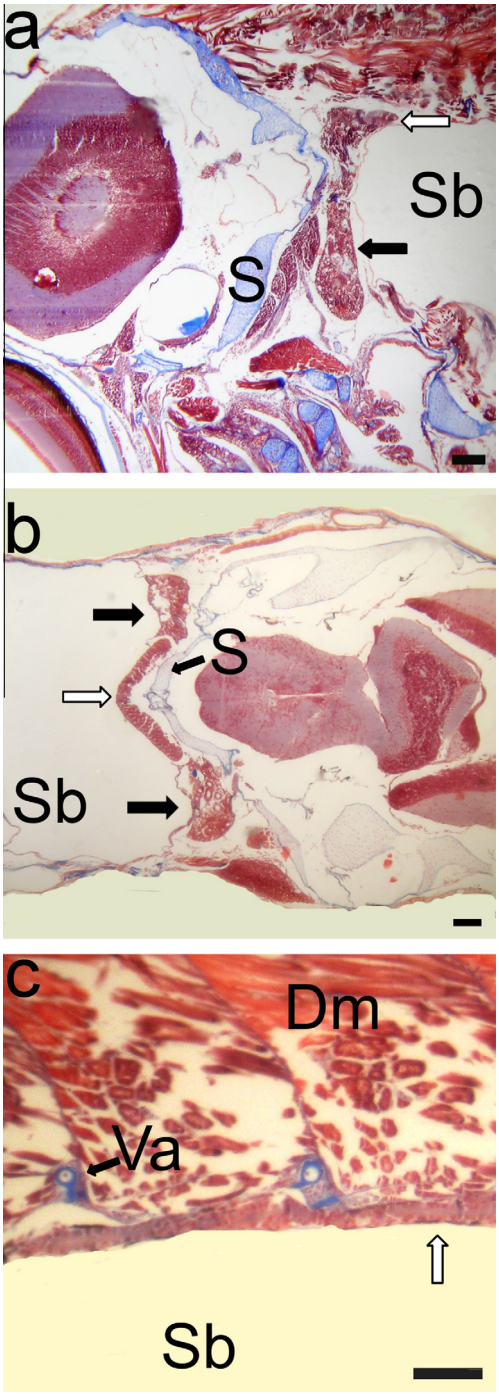

Fig. 2. Juvenile specimens of *Cichlasoma dimerus* aged 45 days. (a) In a parasagittal orientation, it can be appreciated how the cephalic kidney (CK) (black arrow) is located behind the skull (S) and anterior to the swim bladder (Sb); towards the rear portion, it exhibits a dorsal route (white arrow), where the trunk kidney (TK) starts to emerge. (b) In a longitudinal section, the bilateral nature of the CK (black arrows) is evidenced as well as the origin of the TK (white arrow); both portions are behind the skull (S). (c) A higher magnification parasagittal section shows how the TK (white arrow) is located dorsal to the swim bladder (Sb) and in intimate contact with elements of the vertebral column, such as vertebrae apophyses (Va), and associated with the dorsal musculature (Dm). Masson trichrome stain; *scale bar:* 100 μm.

vessels (Fig. 4a). Around these structures, there are small numbers of hematopoietic cells and melano-macrophage centers. With respect to the renal tubules, the most frequently observed are the first proximal segment (PsI), the second proximal segment (PsII) and the distal segment (Ds) (Fig. 4b). The PsI consists of a simple

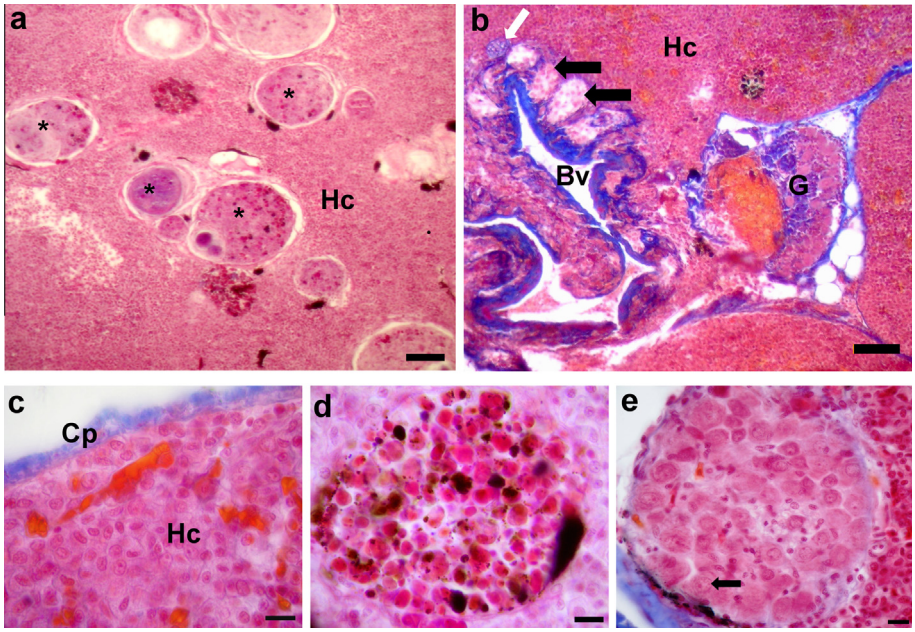

Fig. 3. Cephalic kidney (CK) of adult male *Cichlasoma dimerus*. (a) In the anterior portion of the CK, there is a preponderance of melano-macrophage centers (asterisk) and hematopoietic cells (Hc); PAS stain; *scale bar:* 100 μm. (b) In the posterior portion of the CK, the hematopoietic cells (Hc) are still predominant, and other structures appear, such as blood vessels (Bv), nerve ganglia (G), amyelinated fibers (white arrow) and components of the interrenal gland (black arrows); Masson trichrome stain; *scale bar:* 70 μm. (c) The whole CK is surrounded by a thin capsule of densely packed connective tissue (Cp); Masson trichrome stain; *scale bar:* 10 μm. (d) When stained with PAS, the presence of neutral glycoconjugates in melano-macrophage centers is revealed; *scale bar:* 10 μm. (e) Nerve ganglia are quite common in the posterior portion of the CK, where ganglion neurons (black arrow) can be recognized; Masson trichrome stain; *scale bar:* 10 μm.

cubical to cylindrical epithelium, with a positive PAS reaction in the apical portion of the cells. The cytoplasm is acidophilic with some subnuclear basophilic structures. The nucleus is ovoid, basal and heterochromatic. The PsII has a simple high cubical epithelium, with a reduced lumen when compared to the PsI, but its external diameter is greater; in addition, its cells are wider. Like the PsI, the apical domain is positive for PAS reaction. The nucleus is spherical and located in the center of the cell. This segment is the one most frequently seen in the microscopic fields. Unlike the proximal segments, the Ds has no positive PAS reaction. It consists of a simple cylindrical epithelium with an extensive tubular lumen; the cytoplasm is acidophilic and the nucleus is spherical

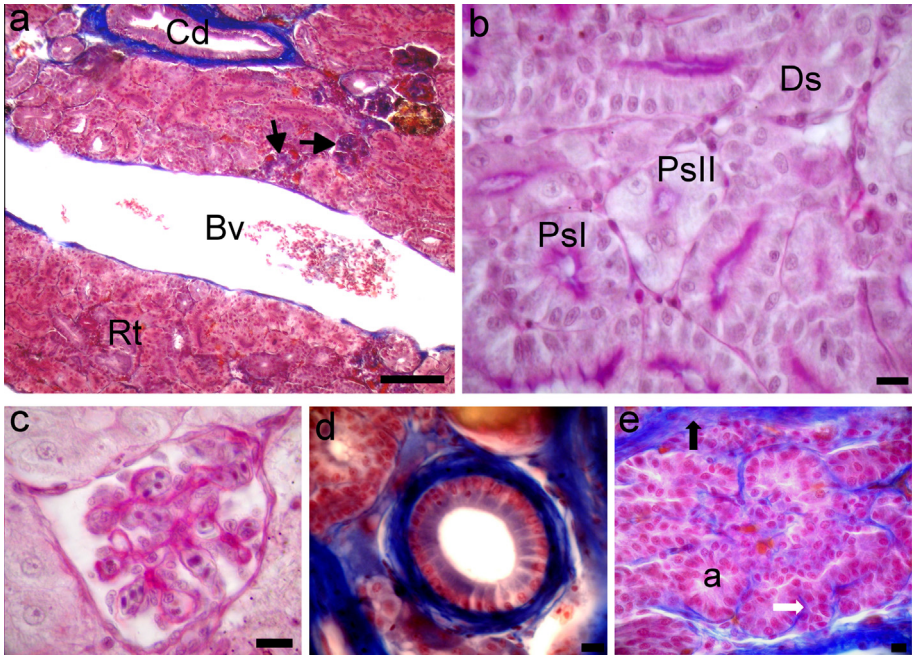

Fig. 4. Trunk kidney (TK) of adult male *Cichlasoma dimerus*. (a) In the TK, the hematopoietic tissue gradually decreases towards the posterior portion, while a great number of renal corpuscles (black arrows), renal tubules (Rt) and collecting ducts (Cd) appear, as well as large blood vessels (Bv); Masson trichrome stain; *scale bar:* 100 μm. (b) The most frequent renal tubules in the visual fields of the TK are: first proximal segment (PsI), second proximal segment (PsII) and distal segment (Ds); PAS stain; *scale bar:* 10 μm. (c) Renal corpuscle; PAS stain; *scale bar:* 10 μm. (d) Collecting duct; Masson trichrome stain; *scale bar:* 10 μm. (e) One or two corpuscles of Stannius are present in the anterior portion of the TK; they are surrounded by a capsule of densely packed connective tissue (black arrow) which extends trabeculae (white arrow), establishing pseudo lobules (a) formed by endocrine cells; Masson trichrome stain; *scale bar:* 10 μm.

and located in the basal portion of the cell. Renal corpuscles (Fig. 4c) and collecting ducts (Fig. 4d) complete the renal structures.

In this portion of the kidney there are no homologous cells to the interrenal cells, i.e., endocrine cells associated with the major blood vessels have never been observed in the TK. The only endocrine components are found in the anterior portion of the TK, corresponding to one or two Stannius corpuscules (Fig. 4e). These are surrounded by a capsule of densely packed connective tissue which extends trabeculae that determine pseudo-lobular structures where the endocrine cells settle.

3.1.2. Interrenal gland

Interrenal cells are found exclusively in the posterior portion of the CK, in contact with the walls of presumed tributaries – smaller veins and sinusoids – of the posterior cardinal vein, and are arranged in groups or chords, separated from each other and from the parenchyma of hematopoietic cells by a thin layer of connective tissue (Fig. 5).

The steroidogenic cells, recognized by their histological characteristics (e.g., spongy cytoplasm), are grouped in chords of two or three layers, surrounded by sinusoids (Fig. 5a–b and e). Since the structural appearance is a result of the plane of section, in some occasions this arrangement seemed to be missing (Fig. 5d and e). These cells – smaller than chromaffin cells – are polygonal in shape (Fig. 5e–h). The nucleus is mostly spherical and basophilic, exhibiting at least one conspicuous nucleolus. When stained with Masson trichrome, the cytoplasm appears acidophilic and spongy in varying degrees. In this sense, it is possible to distinguish two types of steroidogenic cells: (1) large and polyhedral cells with spongy cytoplasm and clear cellular limits (Fig. 5e–h) and (2) small and irregular cells, with little spongy cytoplasm and fuzzy cellular limits (Fig. 5g).

Chromaffin cells are situated in groups of fewer than 10 cells, just below the main veins (Fig. 5i–l). These cells are larger than the steroidogenic cells and exhibit a pale cytoplasm with very fuzzy cell boundaries; a large basophilic and spherical nucleus with a prominent nucleolus is a salient characteristic of these cells (Fig. 5k

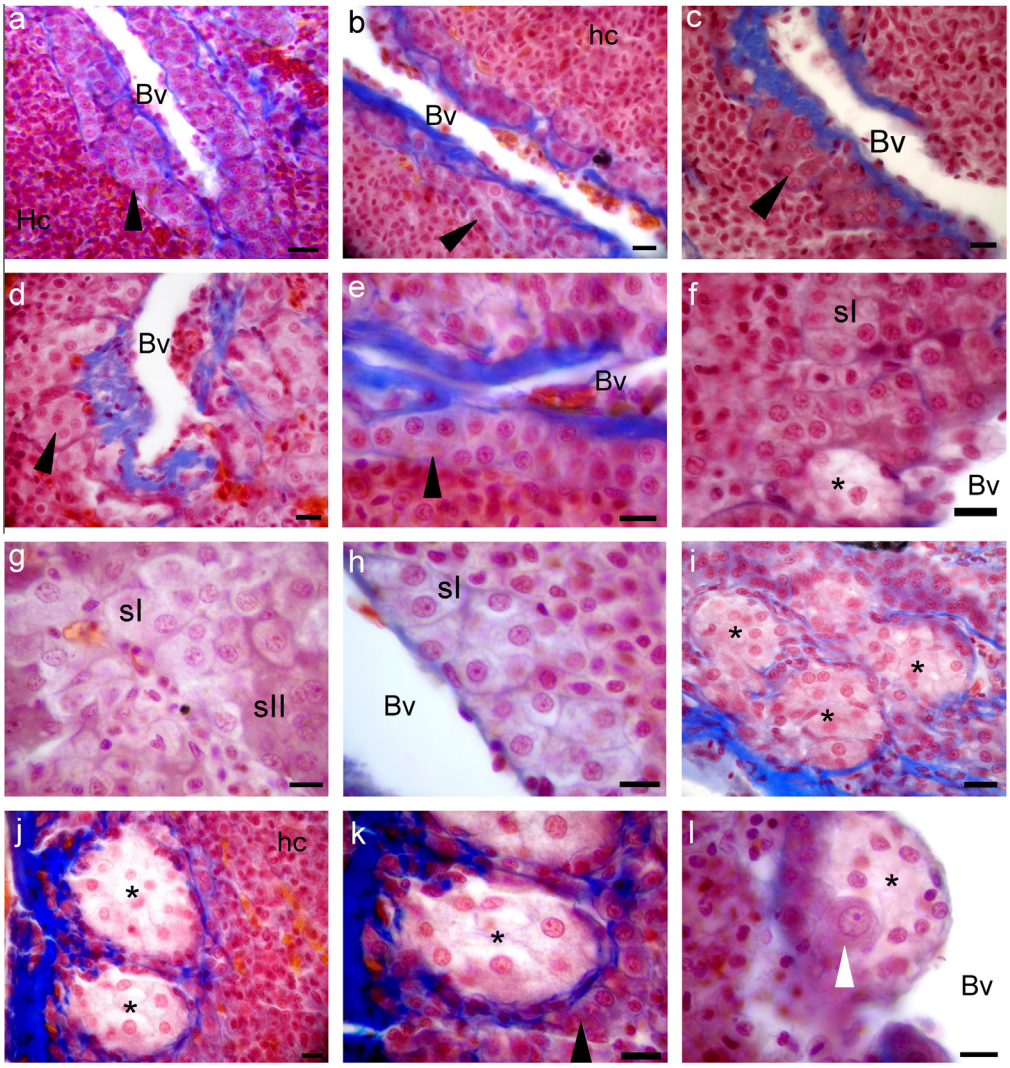

Fig. 5. Interrenal gland in the posterior portion of the cephalic kidney (CK) of adult male *Cichlasoma dimerus*. (a–e) Cells arranged in chords (arrowheads) at the margins of some blood veins (Bv) – post-cardinal vein and their tributaries – surrounded by hematopoietic components (hc) are present in this portion of the CK; these chords are separated from the rest of the parenchyma by a thin layer of connective tissue. (f–h) Steroidogenic cells are arranged in chords; in particular, two types of steroidogenic cells could be detected: type I (sI), with spongy cytoplasm and clear cellular limits and type II (sII), exhibiting a less spongy cytoplasm and diffuse cellular limits. (i–l) Chromaffin cells are arranged in groups (*), also at the margins of blood veins (Bv) and surrounded by a thin layer of connective tissue that separates them from the hematopoietic components (hc); chromaffin cells are larger than the steroidogenic cells (black arrowhead), present fuzzy cellular limits and a pale cytoplasm. In some occasions, chromaffin cells are associated with presumable ganglion cells (white arrowhead). Masson trichrome stain; *scale bar:* 10 μm.

and l). In some situations, they appear to be associated with a presumable ganglion cell (Fig. 5l). There is a close histological association between chromaffin and steroidogenic cells (i.e. Fig. 5f and k).

3.1.3. Immunohistochemical analysis of the kidney

The antibody raised against tyrosine hydroxylase (TH) strongly labeled nervous fibers and cells arranged in groups in the posterior portion of the CK (Fig. 6a and b). Based on the location of these immunoreactive cells and the way in which they are arranged, is very likely that they correspond to the chromaffin cells described in the preceding section.

In the TK, the same antibody labeled nerve ganglion cells structurally different from those described as chromaffin cells (Fig. 6b and c) in the preceding section.

3.2. Relationship among social rank, plasma cortisol and 11-ketotestosterone concentrations and interrenal gland cell morphometry

3.2.1. Organosomatic indexes

No significant differences between T and NT males were found in the indices for spleen (0.08% for T and 0.11% for NT males; $F = 3.08$; $p = 0.096$; $\alpha = 0.025$) and liver (1.58% for T and 1.56% for NT males; $F = 0.002$; $p = 0.967$; $\alpha = 0.05$). Gonadosomatic index also was not significantly different between T (0.122%) and NT (0.081%) males ($F = 3.22$; $p = 0.090$; $\alpha = 0.017$).

3.2.2. Plasma steroid levels and social rank

Plasma 11-KT levels of T males were 2.4 times higher than those exhibited by NT males (107.87 + 9.63 and 44.10 + 5.2 pg/ml, respectively; $F = 10.69$; $p = 0.004$; $\alpha = 0.017$) (Fig. 7a). NT males had plasma cortisol levels 2.2 times higher than T males (261.0 ± 24.4 and 118.5 ± 11.8 ng/ml, respectively; $F = 8.72$; $p = 0.009$; $\alpha = 0.025$) (Fig. 7b).

3.2.3. Social rank and interrenal gland cell morphometry

A relationship between social rank and the morphometry of steroidogenic cells was observed. In particular, cells from NT males had a nuclear area 63.4% larger than T males (28.33 + 1.20 and 17.34 + 0.40 μm^2, respectively; $F = 52.43$; $p < 0.0001$; $\alpha = 0.013$) (Fig. 8a). With respect to chromaffin cells, no significant differences were found in nuclear area between T and NT males ($F = 4.45$; $p = 0.05$; $\alpha = 0.05$) (Fig. 8b).

3.2.4. Correlation between plasma cortisol and 11-KT levels and steroidogenic cell nuclear area

A positive correlation (Pearson coefficient) was found between plasma cortisol levels and the nuclear area of steroidogenic cells ($r = 0.80$; $p < 0.0001$; $\alpha = 0.05$) (Fig. 9a). There was no correlation between plasma 11-KT levels and the nuclear area of steroidogenic cells for T males ($p = 0.76$; $\alpha = 0.05$) (Fig. 9b). For NT males, plasma 11-KT levels correlated negatively with the nuclear area of steroidogenic cells ($r = -0.74$; $p = 0.01$; $\alpha = 0.05$) (Fig. 9b).

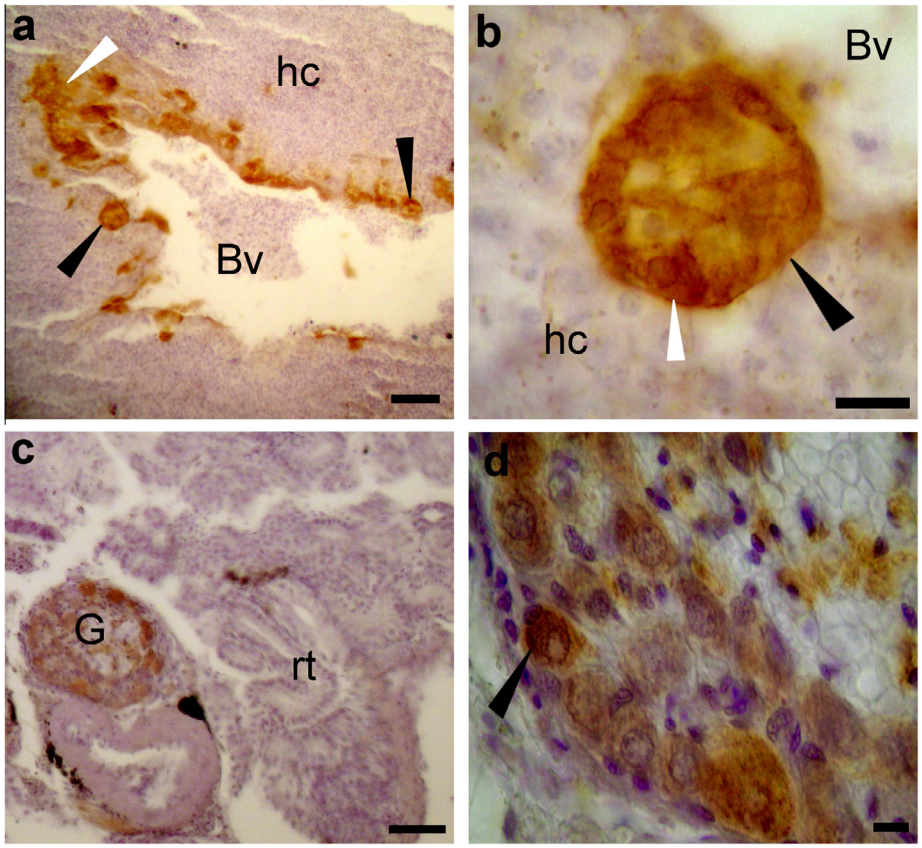

Fig. 6. Immunohistochemical localization of catecholamine-producing cells in the kidney of adult male *Cichlasoma dimerus* using anti-tyrosine hydroxylase (antibody), counterstained with hematoxylin. (a) In the posterior portion of the CK, nervous fibers (white arrowhead) and groups of cells (black arrowheads) located at the margins of blood vessels (Bv) and surrounded by hematoietic components (Hc) were positively stained; *scale bar:* 50 μm. (b) The cells arranged in groups (black arrowhead) in the posterior portion of the CK corresponded to chromaffin cells (black arrowhead); Hc: hematopoietic component; *scale bar:* 10 μm. (c) In the TK, with a predominance of renal tubules (rt), there was a positive stain in nerve ganglia (G); *scale bar:* 80 μm. (d) Cells in (c) corresponded to ganglion cells (black arrowhead); *scale bar:* 10 μm.

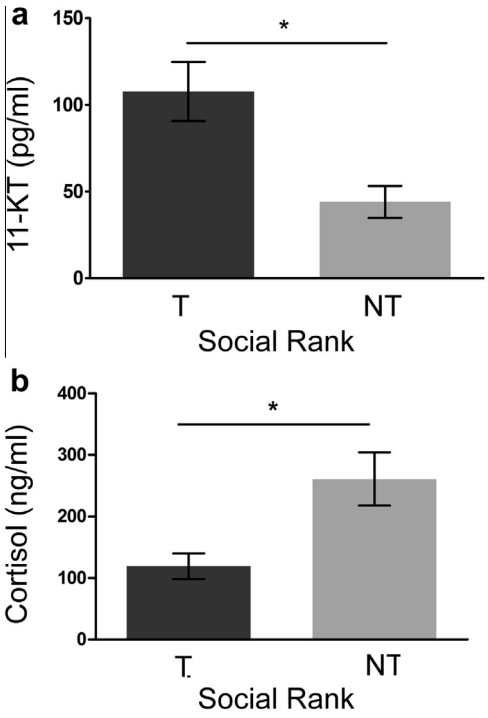

Fig. 7. Mean (±SEM) (a) plasma 11-ketostestoterone (11-KT) ($p = 0.004$) and (b) cortisol concentrations ($p = 0.009$) of *Cichlasoma dimerus*' territorial males in pre-spawning stage (T) and non-territorial males of lowest social rank (NT) ($n = 10$ for each) from community aquaria with established social hierarchies. Asterisk indicates a statistical difference.

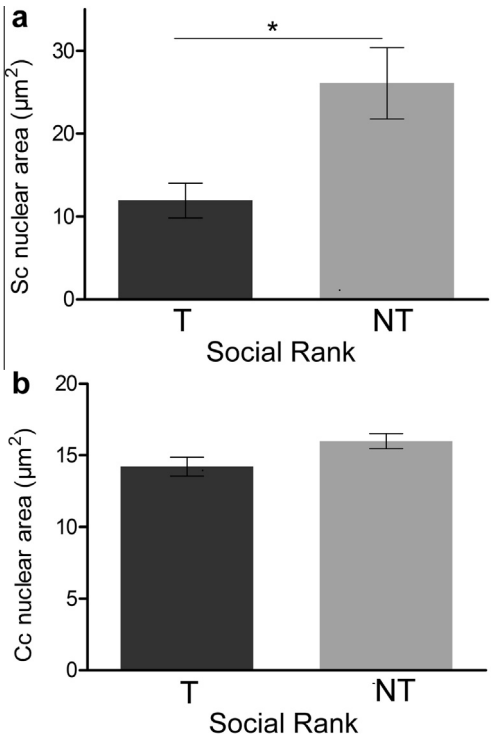

Fig. 8. Nuclear area of (a) steroidogenic (Sc) ($p < 0.0001$) and (b) chromaffin cells (Cc) ($p = 0.05$) of *Cichlasoma dimerus* territorial males in pre-spawning stag (T) and non-territorial males of lowest social rank (NT) ($n = 10$ for each) from community aquaria with established social hierarchies. For each individual, 15 steroidogenic and chromaffin nucleus were measured. Asterisk indicates a statistical difference.

4. Discussion

This work provides an initial characterization of the anatomy and histology of the interrenal gland and its context (the cephalic and trunk kidney) in *C. dimerus*. It also reveals relationships among the plasma levels of two major hormones (cortisol and 11-KT), steroidogenic cell morphometry and social rank in adult male *C. dimerus*.

4.1. The interrenal gland and its context

In *C. dimerus*, the kidney is composed of two structures that are distinguishable by anatomical, histological and functional means. The cephalic portion of the kidney (CK) is bilateral and fulfills three essential functions: (a) hematopoietic, with large numbers of mature and immature hematopoietic cells; (b) immunological, predominating melano-macrophage centers; and (c) endocrine, where the interrenal gland is composed of two main types of cells: chromaffin and steroidogenic. The trunk kidney (TK) plays an excretory and endocrine role (corpuscles of Stannius).

The CK is considered an organ that is similar in terms of function to the mammalian bone marrow (Tomonaga et al., 1973), since it is the principal hematopoietic organ in teleosts (Rombout et al., 2005). Presumably, the wide range of hematopoietic cells observed is not only due to its correspondence with distinct cell lineages – erythrocyte, granulocyte, lymphocyte and thrombocyte – but also with differences in their degree of maturation. Melano-macrophage centers are formed when macrophages aggregate in these structures as they phagocytize heterogeneous materials such as cell residues, melanin pigments and granules of lipofuscin and hemosiderin (Agius and Agbede, 1984).

Tetrapod vertebrates possess a discrete adrenal gland on the anterior side of the kidney (Grassi Milano, 1993) and which is composed of steroidogenic and chromaffin cells. In *C. dimerus*, the interrenal gland components were found exclusively within the posterior portion of the CK, arranged in a relatively diffuse manner with respect to the rest of the parenchyma. Two main cell types were found, steroidogenic cells and chromaffin cells, both in close association with the walls of the posterior cardinal vein, its tributaries and sinusoids.

Chromaffin cells were large, irregular in shape and exhibited a fuzzy cytoplasm. Immunohistochemical analysis revealed that these cells were arranged in clusters. On some occasions, they appeared close to ganglionar cells and unmyelinated nerve fibers. These features have also been described in other teleost species (Rocha et al., 2001).

Steroidogenic cells were smaller than chromaffin cells, cubical, cylindrical or polyhedral in shape, with spongy cytoplasm, usually arranged in chords of one, two or more cells. These characteristics have been observed in most teleost species (Nandi, 1962). With the histological stains used, we were able to distinguish two types of steroidogenic cells. At this stage, it was not possible to determine whether they corresponded to the same cell type with different degrees of activity, or, conversely, if both produced different hormones. In the stickleback *Gasterosteus aculeatus*, two types of steroidogenic cell were also found, based on histological characteristics; the authors suggested that they could be related to cyclical changes in the secretory products of one cell, being able to switch between androgens and corticosteroids according to the stimulus being received (Civinini et al., 2001). Further studies will be required, such as transmission electron microscopy or immunohistochemistry against cortisol or androgens, to clarify this issue.

The other portion of the kidney, the trunk kidney (TK), is mainly excretory, as evidenced by the high percentage of renal corpuscles and renal tubules. No interrenal components such as those

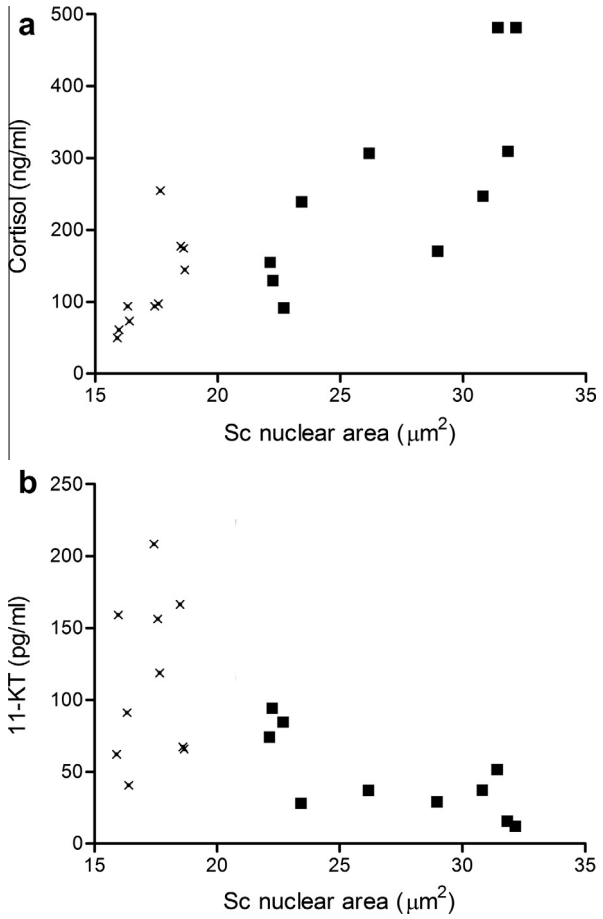

Fig. 9. Relationship between nuclear area of steroidogenic cells (Sc) and (a) plasma cortisol and (b) plasma 11-ketotestosterone (11-KT) levels of *Cichlasoma dimerus* territorial males in pre-spawning stage (T, crosses) and non-territorial males of lowest social rank (NT, squares) ($n = 10$ for each) from community aquaria with established social hierarchies. Plasma cortisol levels and steroidogenic cell nuclear area were positively correlated when all males were pooled ($r = 0.80$; $p < 0.0001$). There was no correlation between plasma 11-KT levels and steroidogenic cell nuclear area for T males ($p = 0.76$), but a negative relationship appeared for NT males ($r = -0.74$; $p = 0.01$).

described in the posterior portion of the CK were found in the TK. However, in other Perciformes, such as *Dicentrarchus labrax* or *Sparus aurata*, chromaffin cells were found near the walls of the great vessels within the TK (Grassi Milano et al., 1997), while steroidogenic cells were never found.

By means of the immunohistochemical analysis, we found cells immunoreactive cells tyrosine hydroxylase (TH) in the TK. These cells could be producing adrenaline, noradrenaline and/or dopamine, since TH is necessary for their biosynthesis (Kaufman, 1995). Some authors suggest that these cells are part of sympathetic ganglia within the surface of the TK and that they must be included as components of the interrenal gland (Grassi Milano et al., 1997). In *C. dimerus*, these cells were, indeed, components of sympathetic ganglia within the TK. There is controversy as to whether all chromaffin cells found in an organism, regardless of their location, should or should not be considered the same elements of the interrenal gland (Youson, 2007). Since the histological characteristics between these cells and those found in the posterior portion of the CK were different in terms of their shape, size and disposition, they cannot be considered the same as those found in the latter portion. Furthermore, independently of the hormone that they produce, the synthesis and release may be differently regulated.

With regard to the excretory component of the TK, the nephron was organized as follows: (1) renal corpuscle, (2) first proximal segment, (3) second proximal segment (4) and distal segment. Besides these components, in most of the freshwater teleost fishes there is a very short intermediate segment between (3) and (4) and a ciliated neck that emerges from the renal corpuscle (Yasutake and Wales, 1983). These segments were not found in the TK of *C. dimerus*, presumably because their presence was sporadic in the visual fields. The renal corpuscle was large and highly vascularized, with prominent glomerulus, features that have already been reported in most freshwater teleosts (Trump et al., 1975). The tubules exhibited a great variability, mainly in terms of size, amount of connective tissue, presence or absence of isolated smooth muscle cells and epithelial type, histological features found in most freshwater teleost fish studied to date (Sakai, 1985).

One or two corpuscles of Stannius appeared within the anterior portion of the TK. These endocrine structures, whose main product is stanniocalcin, a hypokalemic hormone (Youson, 2007), is absent in some teleost species (Wendelaar Bonga and Pang, 1991).

4.2. Interrenal gland and the establishment of social hierarchies

The behavior exhibited by dominant males includes selection of a territory and aggression directed towards animals of lower social rank (Parikh et al., 2006; Sloman et al., 2000). By contrast, subordinates often are excluded from access to food (McCarthy et al., 1992), and show less aggressive behavior (DiBattista et al., 2005). In *C. dimerus*, plasma levels of cortisol and 11-KT were 2.2 times lower and 2.4 higher, respectively, in T males (dominant/territorial pre-spawning males) with respect to NT males (males of lowest social rank). Thus, plasma levels of these hormones were related to social rank, but we cannot establish whether these levels were a cause or consequence of status under our experimental design.

The physiological correlates of stress in cichlids of different social ranks has been extensively assessed. In particular, Fox et al. (1997) found that non-territorial males of *A. burtoni* had significantly higher cortisol levels than territorial ones, results consistent with previous findings in *C. dimerus* (Alonso et al., 2011). An opposite pattern has been observed in a cooperatively breeding species, such as the cichlid *Neolamprologus pulcher*, where dominants exhibited higher cortisol levels than subordinates (Mileva et al., 2009), and in the territorial cichlid Nile tilapia (*Oreochromis niloticus*) where cortisol levels are similar in subordinate and dominant males (Correa et al., 2003). Lower plasma concentrations of cortisol in subordinate rainbow trout (*Oncorhynchus mykiss*) (Øverli et al., 1999) have been correlated with an increase in the corticotropin-releasing factor mRNA in the preoptic area of the brain (Doyon et al., 2003) and in plasma levels of adrenocorticotropic hormone (Höglund, 2000).

Few studies have evaluated the morphometry of interrenal gland cells in animals of different social rank: Noakes and Leatherland (1977) found that after 14–17 days of interaction between male rainbow trout, steroidogenic cells of subordinates showed greater synthetic activity – estimated in terms of the nuclear area – with respect to the dominant ones. Similar results were obtained by Scott and Currie in subordinate males of *Xiphophorus helleri*, although they used the nuclear diameter instead of its area (Scott and Currie, 1980). The higher plasma cortisol levels exhibited by *C. dimerus* NT males in the present study were consistent with the increased activity (estimated through the nuclear area) found in their steroidogenic cells, where a 63.4% larger nuclear area was observed compared to dominant T males. In addition, a positive correlation was found between plasma cortisol level and the nuclear area of steroidogenic cells. Taken all together, these results suggest that those males may have been in a condition of chronic stress. The lack of difference in the nuclear area of chromaffin cells

between T and NT males may be due to the fact that, while catecholamines increase during acute stress, they decrease in chronically stressful conditions (Sumpter, 1997). Given that there were significant differences in plasma cortisol levels and the nuclear area of the steroidogenic cells between T and NT males, and that both parameters were positively correlated, the morphometry of the interrenal gland cells could be used as an indicator of social rank in these fish, provided that chronic stress is correlated with male's the position in the social hierarchy. In addition, differences in the interrenal gland cell morphometry may be considered as evidence of chronic stress in small teleosts, where is difficult to obtain plasma samples for measuring cortisol. It is important to note that in teleost fish, as well as in most vertebrates, cortisol is produced exclusively in the steroidogenic cells of the interrenal/adrenal gland (Mommsen et al., 1999).

In a situation of social instability, where social rank is dependent on agonistic encounters, one would expect to find higher plasma levels of androgens in dominant males, especially in those species were aggressive competition is important for rank acquisition (Parikh et al., 2006). Once the social hierarchy was established, plasma levels of 11-KT, a hormone that correlates with aggression in males of several species of cichlid fishes (Hirschenhauser et al., 2004), were higher in T males. It is known that elevated plasma levels of cortisol inhibit the synthesis of 11-KT (Consten et al., 2001) and, thus, increased levels of cortisol in the NT males may be at least a partial explanation for their reduced plasma 11-KT levels. Moreover, a negative correlation was found between the nuclear area of steroidogenic cells and 11-KT plasma levels exclusively in NT males. Consten et al. (2002) have proposed that, in the teleost fish *Cyprinus carpio*, cortisol sensitivity depends on the reproductive maturational status of the animal. It is probable, however, that in the *C. dimerus*' NT males, but not in T males, cortisol levels reached the threshold to produce a concentration-dependent decrease in plasma 11-KT levels.

During chronic stress situations, behavioral and physiological attributes related to reproduction are inhibited to a greater or lesser extent, redirecting resources to other organs. In humans, for example, during stress, there is a decrease in the blood flow to the testes (Kraut et al., 2004). In stable groups of the cichlid fish *N. pulcher*, dominant individuals had higher gonadosomatic indexes (GSI) (Fitzpatrick et al., 2006). In our experimental design, no differences in GSI were observed between T and NT males, although there was a trend towards higher GSI's in T males. If cortisol inhibits the synthesis of 11-KT, the most potent androgen in male teleost fishes, it raises the question of why such differences in the GSI were not observed. Subordinate male teleosts, although being socially inhibited, are often not reproductively incompetent; they retain some activity at all levels of the reproductive axis, from the brain to the testes. This gives the subordinate male a physiological substrate for reproduction, in case a "social ascent" opportunity appears (Maruska and Fernald, 2010). This could also be occurring in *C. dimerus*, where social hierarchies are highly dynamic and thus the possibility of social ascent is also present. On the other hand, the GSI may not be providing relevant functional information, such as cellular composition (Maruska and Fernald, 2010). In order to clarify this issue, future studies should include characterization of testicular histology.

In this work, it we showed that *C. dimerus* males of lowest social rank (NT) of exhibited higher plasma levels of cortisol, lower plasma levels of 11-KT and an increased activity (estimated through the nuclear area) of their steroidogenic cells compared to the territorial/dominant pre-spawning (T) males, all facts suggesting that NT males were subjected to a condition of chronic stress. Because nuclear area of the steroidogenic cells positively correlated with plasma cortisol levels, the morphometry of the interrenal gland cells may be useful as an additional indicator of stress in fish. In this sense, future studies will be required for a better understanding of the morphology of the interrenal gland and other studies will be necessary to elucidate the physiological mechanisms underlying reproductive modulation due to high plasma cortisol levels.

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