

Social and reproductive physiology and behavior of the Neotropical cichlid fish *Cichlasoma dimerus* under laboratory conditions

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In this work we describe for the first time the social and reproductive behavior of the Neotropical fish *Cichlasoma dimerus* (Heckel, 1840) [Perciformes: Cichlidae], endemic to the Paraná River basin, using a comprehensive-integral approach, including morphological and physiological features. This substrate breeding fish has biparental care of the fry and presents a dominance hierarchy that determines access to breeding territories among males, and to males with territories among females. Gregarious behavior associated with a pale body color, was observed before reproductive behaviors started. Afterwards, a dominance hierarchy was established through aggressive interactions. Territorial individuals had bright body color patterns and non territorial an opaque grey one. Black ventral coloration was associated with reproductive individuals. Courtship displays, which were similar to threatening displays, had the common effect of increasing the visible area of the individual. The dominant male was always the largest one suggesting that size is probably a major factor determining the hierarchy establishment and that these intra-sexually selected traits may have been reinforced by inter-sexual selection. Reproductive males had higher pituitary levels of β -follicle stimulating hormone (β -FSH) and somatolactin (SL) than non reproductive ones, while no differences were found among females. No differences were found among male gonadosomatic indexes. Non reproductive individuals had higher plasma cortisol levels for both sexes. It is possible that dominant reproductive individuals may be inhibiting reproduction of subordinate fish through physical contact, increasing their cortisol levels and diminishing FSH and SL pituitary content. However, this was not reflected as an inhibition at the gonadal level in our experimental design.

En este trabajo se describen por primera vez el comportamiento social y reproductivo del pez cíclido neotropical *Cichlasoma dimerus* (Heckel, 1840) [Perciformes; Cichlidae], endémico de la cuenca del Paraná, desde un enfoque integral y abarcador, incluyendo características morfológicas y fisiológicas. Éste pez incubador de substrato, tiene cuidado biparental de las crías y presenta una jerarquía de dominancia que determina el acceso a territorios reproductivos entre los machos, y a machos con territorios entre las hembras. Se observó un comportamiento gregario con una coloración corporal pálida característica, antes que comenzaran los comportamientos reproductivos. Luego, una jerarquía de dominancia se estableció a través de interacciones agresivas. Los individuos territoriales presentaron patrones de coloración corporal brillantes y los individuos no territoriales uno gris opaco. Una coloración ventral oscura fue observada asociada a individuos reproductivos. Los despliegues de cortejo fueron similares a los de amenaza y tuvieron la característica común de aumentar el área visible de los peces. El macho dominante fue siempre el más grande, sugiriendo que probablemente la fuerza (tamaño) es un factor preponderante determinando el establecimiento de las jerarquías y que éstas características seleccionadas intrasexualmente pueden haber sido reforzadas por selección intersexual. Los machos reproductivos presentaron un mayor contenido hipofisario de β -FSH y SL que aquellos no reproductivos, mientras que no se encontraron diferencias entre las hembras. No se encontraron diferencias entre los índices gonadosomáticos de los machos. Los individuos no reproductivos presentaron niveles plasmáticos mayores de cortisol para ambos sexos. Aunque los individuos reproductivos dominantes podrían estar inhibiendo la reproducción de los peces menos dominantes a través de interacciones de contacto físico, aumentando sus niveles de cortisol y disminuyendo el contenido hipofisario de FSH y SL, esto no se vería reflejado a nivel gonadal en nuestro diseño experimental.

Key words: Cichlidae, Cortisol, Gonadosomatic index, Gonadotropins, Somatolactin.

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Introduction

Among social animals, dominance can have long-term physiological consequences. There are strong interactions between behavior, the endocrine and nervous systems that affect the interactions between an animal and its environment, including social interactions. For example, dominance status has been shown to control fertility (Fraley & Fernald, 1982; Abbot *et al.*, 1998; Faulkes & Bennet, 2001), suppressing gonadal maturation in subordinate individuals (Barret *et al.*, 1990), and also influence on neurogenesis (Kozorovitskiy & Gould, 2004), growth rate (Hofmann *et al.*, 1999), and stress (Fox *et al.*, 1997; Abbot *et al.*, 2003). Although social regulation on several physiological processes is a well-established phenomenon, little is known about the mechanisms linking the social environment to physiological changes associated with dominance. Fox *et al.* (1997) found that the level of the stress hormone cortisol depends critically on both the social and reproductive status of an individual fish and on the stability of its social situation.

Many teleost fish exhibit a wide range of behavioral responses to environmental and social stimuli, which are quite often associated with their reproduction. Reversible changes in reproductive competence, adoption of distinct reproductive strategies within one sex, and reversible or permanent changes in gonadal sex are examples of the influence of the behavior on the reproduction (Ross, 1990; Taborsky, 1994). In this context, cichlids (Perciformes: Cichlidae) have been intensively used as experimental models for studies of social control of reproduction, in particular African cichlids, such as the African tilapia *Astatotilapia burtoni* (Günther, 1894) (= *Haplochromis burtoni*) and *Oreochromis mossambicus* (Peters, 1852) (Fox *et al.*, 1997; Ogawa *et al.*, 2006). Behavior of African cichlids is better characterized than the Neotropical cichlids, and most of whose species have not been extensively studied at the behavioral level. Although the Convict cichlid, *Amatitlania nigrofasciata* (Günther, 1867) from Central America is the most studied species at the behavioral level (Noonan, 1993; Nuttall & Keenleyside, 1993; Wisenden, 1995), South American cichlids have not been studied yet with respect to the physiological processes triggered by different behavior responses, and also regarding the intraspecific social interactions effects on reproduction. *Cichlasoma dimerus* (Heckel, 1840) is a South American cichlid that easily adapts to aquarium conditions and spawns with high frequency (every 15 days approx.) during 6 months of the year under natural conditions and throughout the year in the laboratory under long photoperiod (14 h) and controlled temperature (20–26°C). Due to these advantageous characteristics, *C. dimerus* is becoming an interesting model for reproductive and developmental studies (Pandolfi *et al.*, 2009). In addition, *C. dimerus* displays an elaborate social system and complex, relatively stereotyped breeding activities, which make this species very suitable for studies of intraspecific social interactions on reproduction.

The aim of this study is to describe social and reproductive behavior of *C. dimerus* under laboratory conditions. Based on the hypothesis that non reproductive individuals may be inhibited at some level of the hypothalamic-pituitary-gonadal axis, we have evaluated reproductive parameters, such as gonadosomatic index, plasma cortisol levels and pituitary hormones content (β -LH and β -FSH semiquantification) within the different social statuses of *C. dimerus*. In addition we have analyzed body color and somatolactin (SL) pituitary content within the different social statuses. Previous studies on this species and in the Red Drum (*Sciaenops ocellatus*) showed an effect of SL on body color background adaptation (Zhu & Thomas, 1996; Cánepa *et al.*, 2006). Also, between resting and sexually active females of Pejerrey (*Odontheistes bonariensis*) differences were found in the area of immuno reactive (ir) SL cells, and also a positive correlation between the ir-SL cellular area and the gonadosomatic index. In males, this correlation was not found (Vissio *et al.*, 2002). Preliminary observations in *C. dimerus* showed differences in body color related to different social status. Therefore, we decided to monitor body color in relation to different social status and also measure pituitary SL content.

Material and Methods

Animals

Cichlasoma dimerus caught in Esteros del Riachuelo: 27°35'S 58°45'W (Corrientes, Argentina) were housed in aquaria under conditions mimicking their natural habitat (Casciotta *et al.*, 2005; Alonso, pers. observ.) for at least 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 (*Cabomba* sp., *Ceratopteris* spp., *Echinodorus* sp., and *Egeria densa*) 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). All the experiments were conducted in accordance with international standards on animal welfare as well as being compliant with local and national regulations. Procedures are in compliance with the National Institutes of Health Guide for Care and Use of Laboratory Animals (NIH publication no. 85-23, revised in 1985). In some experiments, when aggressive interactions appeared to be excessive, the animals were separated to avoid injuries.

Experimental design

Under controlled conditions of temperature and photoperiod described above 4 females and 4 males of *C. dimerus*, size matched, were kept in an aquarium (density = 50 individuals/square m) with an internal mechanical filter. The aquaria was divided in the middle by a barrier of rocks and plants and it had two flat dark stones over the gravel, one on each side (*C. dimerus* has a preference for this kind of surface for laying their eggs) (Fig. 1). Animals were observed

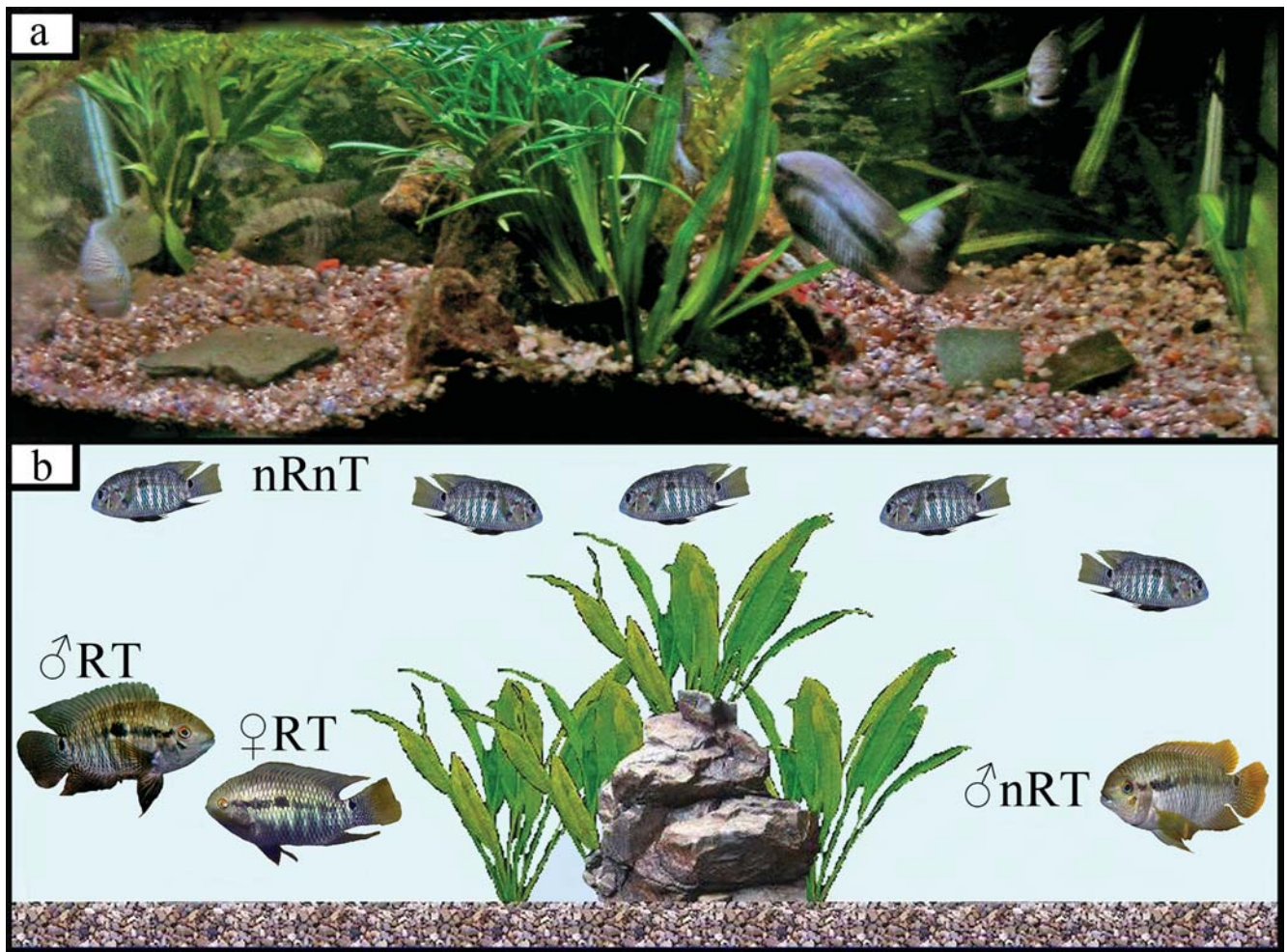


Fig. 1. Distribution of different social status fish on the experimental aquaria: **a)** Picture of the experimental aquaria; **b)** Schematic representation. Abbreviations of social statuses used: nRnT = non Reproductive non Territorial individuals; nRT = non Reproductive Territorial individual; RT = Reproductive Territorial individual. For a detailed description of these social statuses see Table 3.

daily for periods of at least 15 min in the morning and in the afternoon to verify social status, color pattern and general behavior. The establishment of territories, social dominance hierarchies, and reproductive behavior were observed. Larvae hatched from eggs two days after fertilization, and started to swim eight days after fertilization (Meijide & Guerrero, 2000). When the most aggressive interactions between the dominant pair and the subordinate individuals were registered, we expected to find the most significant physiological differences among the groups. Therefore, animals were sacrificed 8 days post-spawning of the reproductive pair (RTm and RTf).

Only 4 out of the total 8 fish in each aquarium were sampled at the end: 1 dominant pair (1 male and 1 female, Reproductive Territorial [RT] individuals), and 2 subordinate individuals (1 non Reproductive non Territorial [nR-nT] male and 1 nR-nT female) were sacrificed per experiment. The nR-nT individuals used here were always those ones in the lowest position in

the social hierarchy. The animals were anesthetized by immersion in a 0.1% benzocaine solution. Animals were weighed and standard length measured. Blood samples were taken to measure cortisol plasma levels and afterwards animals were sacrificed by decapitation. Testes were weighed to determine the gonadosomatic index. Pituitaries were processed as described below for Western blot analysis. The experiment was repeated 4 times (4 replicates) using a total of 32 individuals ($N = 4$; $n = 32$).

Plasma cortisol measurement

Blood was drawn from fish immediately after capture (less than 4 min) by caudal puncture and stored in heparinized tubes to allow it to clot. Then, serum was separated by centrifuging the samples at 3000 rpm for 20 min and stored at “20°C until assay. Cortisol assay was measured by ECLIA (electrochemiluminescence immunoassay) using a Cobas analyzer (Roche).

Semiquantification of β -FSH, β -LH, and SL pituitary content

In order to semi-quantify pituitary content of hormones related to reproduction (β -FSH, β -LH and) and background color adaptation (SL), an analysis with 15% sodium dodecylsulfate-polyacrylamide gel electrophoresis (SDS-PAGE) followed by Western blot was performed. The hormones were detected using heterologous antisera, the specificity of which had been previously tested in this species (Pandolfi *et al.*, 2001, 2006) (Table 1). After dissection, each pituitary was homogenized in 100 μ l of Tris-HCl buffer 50mM, pH 7.4, with 1 μ l of protease inhibitor cocktail (Sigma, St Louis, Mo.). Then, 15 mg of protein with loading buffer (120mM Tris-HCl pH 6.8, 3% dodecylsulfate, 10% glycerol, 1% β -mercaptoethanol) were heated at 100°C for 5 min and loaded into the SDS-PAGE. 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 Tris Buffer Saline Tween20 (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 different primary antisera (Table 1) 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 semi-quantification was performed using Image Gauge version 3.12 (Fuji Photo Film) software. In order to avoid a possible loading error in the SDS-PAGE, pituitary hormone content was semi-quantified by densitometric analysis and normalized to the optical density obtained for α -tubuline.

Statistical analysis

Statistical analysis of cortisol data was performed using a three factor analysis of variance (ANOVA), with two fixed factors: sex and reproductive state; and one random factor: experiment repetition. This design allowed us to test for interactions between sex and reproductive state and also to control some experimental error regarding to variability among experiment repetition. Statistical analysis of organ-somatic index was performed by using Student's t-test. Statistical analysis of Western blot data was performed using the Mann-Whitney test. All suppositions for these statistical analyses were tested. Statistical significance was established at the $p < 0.05$ level for all tests. Data was presented as means \pm SEM. Statistica 7.0 Software (Statsoft, Inc) was used for analysis.

Results

We observed that the commonly used categories of "Dominant" and "non-Dominant" commonly used for the social status of individuals were not suitable for *C. dimerus*

Table 1. Antisera used in the Western blot analysis and estimated molecular mass of *Cichlasoma dimerus* pituitary hormones. ¹Departamento de Bioquímica y Biología Molecular, Facultad de Ciencias, Universidad de Cádiz, Spain; ²National Research Institute of Fisheries Science. Fisheries Research Agency. Kanazawa, Yokohama, Japan.

Antisera	Dilution	Source	Donated from	Detected ir-band/s
anti-SL	1:2000	<i>Sparus aurata</i>	Antonio Astola ¹	32 & 28 kDa
anti- β FSH	1:1000	<i>Fundulus heteroclitus</i>	Akio Shimizu ²	19 & 15 kDa
anti- β LH	1:2000	<i>Fundulus heteroclitus</i>	Akio Shimizu ²	24 kDa

because dominance in this species seems to be linear (A>B>C>D...) rather than despotic (A>B=C=D...). For example, A would be dominant with respect to B, C, and D and B is dominant with respect to C and D but also is a subordinate to A. This ambiguity made these categories not adequate to describe the different social status observed in *C. dimerus*. Therefore, we propose new categories that better explain the social status observed, based on the reproductive state and territoriality of individuals (Table 2).

Sexual dimorphism

Although there are no conspicuous external morphological differences observed between males and females, in general *C. dimerus* males are larger than females (10 cm and 49 g vs. 8 cm and 32 g respectively; standard length (SL) and mass). Mean female sl is about 84% that of males. The larger males (SL>9 cm) sometimes presents some of the posterior dorsal fin rays elongated and curved, in hooked shape to the posterior margin of the fin. These elongate rays often extended beyond the caudal fin distal margin as seen in the male of Fig. 2e. Remarkably, some males, especially smaller ones (SL<9 cm), do not have this curved margin (Fig. 2c) and some females can also have relatively elongated dorsal fin rays (Fig. 2d), but the posterior end of the dorsal fin never has the hooked shape described above. Nevertheless, sexes could be differentiated during the spawning period by their distinct body color patterns and

Table 2. Dominance and social statuses in *Cichlasoma dimerus*.

	Social Status
	Gregarious
D o m i n a n c e	RT = Reproductive Territorial
	nRT = non Reproductive Territorial
	nRnT = non Reproductive non Territorial

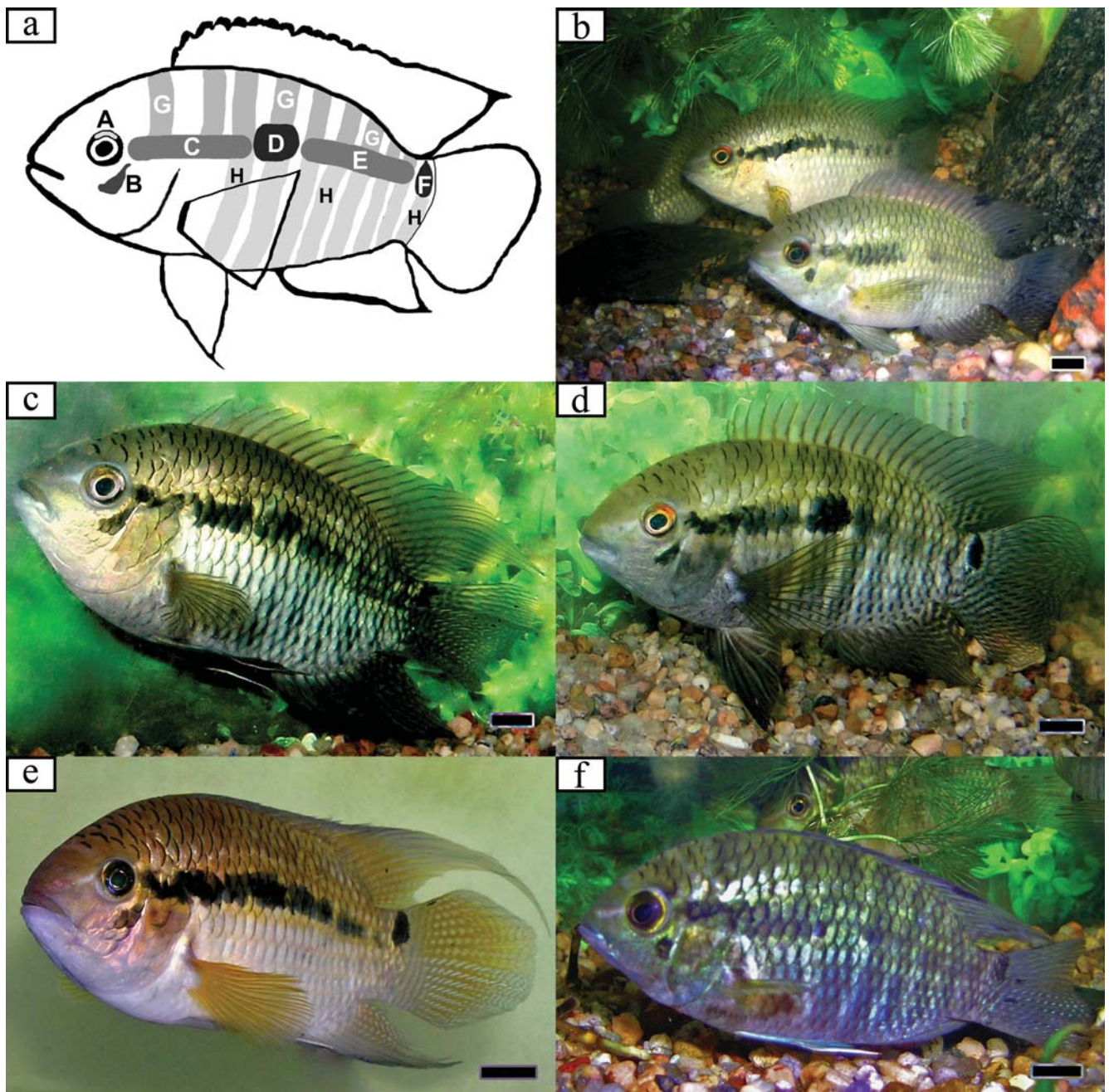


Fig. 2. Body color patterns associated with different social status; **a)** Schematic representation of the main features of *Cichlasoma dimerus* color pattern. A: red eye blotch; B: preopercular blotch, C: anterior longitudinal line; D: mid trunk blotch; E: posterior longitudinal line; F: peduncular blotch; G: dorsal transversal bars; H: ventral transversal bars. These features may “turn on” or “off” in different conditions; **b)** Gregarious individuals; **c)** Reproductive Territorial male (RT); **d)** Reproductive Territorial female. **e)** Non Reproductive Territorial mal (nRnT); **f)** Non Reproductive non Territorial (nRnT) individual. Scale bar = 1 cm

the external morphology of the genital papillae, which was thinner and ending in a point in males vs. wider and blunt in females.

Social behavior: Territory establishment

A schematic representation of the distribution of different social status fish in the experimental aquaria is shown in Fig. 1. The different features of color patterns associated with

different social status are shown in Fig. 2 and Table 3. *Cichlasoma dimerus* displayed different stripes and blotches on their body that “turned on” or “off” in different situations, presumably being mainly under nervous control. Once fish were placed in the experimental aquarium, animals showed a gregarious behavior (by definition gregarious behavior is the tendency to move in or form a group with others of the same kind) with a characteristic pale body coloration pattern during

Table 3. Description of body color patterns observed in different social statuses. Territorial individuals have a bright background color, meanwhile non territorial presents dark background color. Also, reproductive individuals show a darkening of ventral body region, as opposed to the whitish ventral region in gregarious ones.

Social status	Body color pattern description
Gregarious	Pale background color. Grayish dorsal region and whitish ventral region. Transverse stripes may be present but always grey and not very conspicuous.
Reproductive-Territorial male (RT male)	Bright background color may vary from blue, green to yellow. Ventral region and anal fin dark grey. Pectoral fin black. Head ventral portion, below mouth and the opercle section, dark grey. Transverse ventral stripes dark, especially those in the posterior half. The general darkening is intensified during spawning and periods of parental care while it is not very conspicuous in the prespawning period.
Reproductive Territorial female (RT female)	Similar to RT male, although bright background color is generally green and the ventral portion and pectoral fin darkening is generally more conspicuous, especially that of the opercles. This last difference is more evident in the prespawning period, when the female is darker than its mate.
Non reproductive-Territorial (nRT)	Bright background color, similar to the Reproductive individuals (R), although generally not as bright. Differ from R color pattern by not showing the characteristic ventral and anal and pectoral fin darkening. Transverse stripes not conspicuous, may not be present.
Non reproductive-non Territorial (nRnT)	Dark grey background color. Transverse stripes always present. Bright colors never present.

the first day (Fig. 2b). Animals were not very active at this stage and did not show aggressive interactions though, only occasional sporadic aggressive behaviors were observed a few min after fish were first placed in the aquarium. After 24 h, animals started to show explorative and active behavior and many aggressive interactions were observed, which we classified into two types: 1) physical contact and 2) threatening displays. (Table 4, Fig. 3).

Physical interactions (Fig. 3) were generally preceded by threatening displays, especially during establishment of territories and social hierarchies. All these aggressive interactions resulted in a social hierarchy which starts with the territorial delimitation by the most dominant male. In the given experimental conditions, the largest male chose a territory close to the gravel substrate limited horizontally by stones or plants and aggressively defended it from other fish. These males were defined as Reproductive-Territorial male (RTm) (Fig. 1), showing a characteristic body coloration pattern as described above (Fig. 2c). The female mate of this male was called Reproductive Territorial females (RTf) (Fig. 1). At the same time another male defended a territory on the substrate on the opposite side of the aquarium. This male defended its territory from other subordinate fish but was continuously attacked by the dominant pair (RTm and RTf) and did never reproduce. This animal was called as non reproductive-Territorial male (nRTm) and it was easily recognizable not only by its position in the aquarium but also from its body coloration pattern (Fig. 2d). Individuals that could not defend a territory were considered as non Reproductive-non Territorial (nR-nT). They were observed hiding between the floating plant roots and displayed a characteristic dark color pattern (Fig. 2e) and fin retraction. nRT males and nT-nD individuals showed submission displays, such as escape and fin retraction, towards dominant individuals.

Reproductive behavior: Prespawning

Several days after fish were transferred to the experimental aquarium, territories were progressively established. In Fig. 1

a schematic distribution of the different social status individuals in the experimental aquarium is shown. The dominant male aggressively defended the prospective spawning site, which is usually a flat stone on the gravel. They seem to prefer horizontal and smooth hard surfaces of about 100 cm² or more. The male takes stones and cleans the spawning surface with its mouth and undulates its body in an “S” shaped way over the spawning surface. Some females were aggressively rejected from the territory by the male which exhibited bright background coloration (Fig. 2c). The same “threatening displays” described above were also performed towards the females that were eventually accepted as potential mates by the male. Since only one RTm was found in our experimental design, it is still unclear if females can choose between different RTm. When the pair was formed, the Reproductive Territorial female (RTf) (Fig. 1, 2f) defended the

Table 4. Ethogram outlining the aggressive, submissive and reproductive behavioral repertoire of *Cichlasoma dimerus*.

Social Interactions		
Aggressive	Physical contact	Bites
		Tail hit
		Mouth holding
		Fin erection
		Chasing
	Threatening displays	Opercula opening
		Head Shaking
		Body Shaking
Submissive displays		Escape
		Fin retraction
Reproductive displays		Fin erection
		Opercula opening
		Head Shaking
		Body Shaking
		Stone biting
		Wave movements over the spawning surface (SF)
		Genital papillae rubbing on the SF
		S body shape and shaking over the SF
		Pit digging
		Eggs fanning
		Pectoral fin shaking



Fig. 3. Image showing aggressive physical contact between two males, referred to as mouth holding.

territory with the male and both acquired a characteristic color pattern with a darkened grey body ventral region (Fig. 1g). We have also observed in all social statuses that the transverse lines darkened during the aggressive behaviors performed by the individuals; probably this darkening is correlated with aggressiveness as a threatening display.

Reproductive behavior: Spawning and parental care

Cichlasoma dimerus engages in biparental care of the fry. The male and female rubbed their genital papillae over the spawning substrate and cleaned it with their mouths. The male shook over it showing its flanks to the female. The female laid adherent eggs over the spawning surface in a line of around 10 eggs and then the male passed over fertilizing them (Fig. 4a). It was observed that spawning may last up to 90 min and, depending on the size of the female, from 400 to 800 eggs are laid (Fig. 4b). The number of eggs which are laid may be

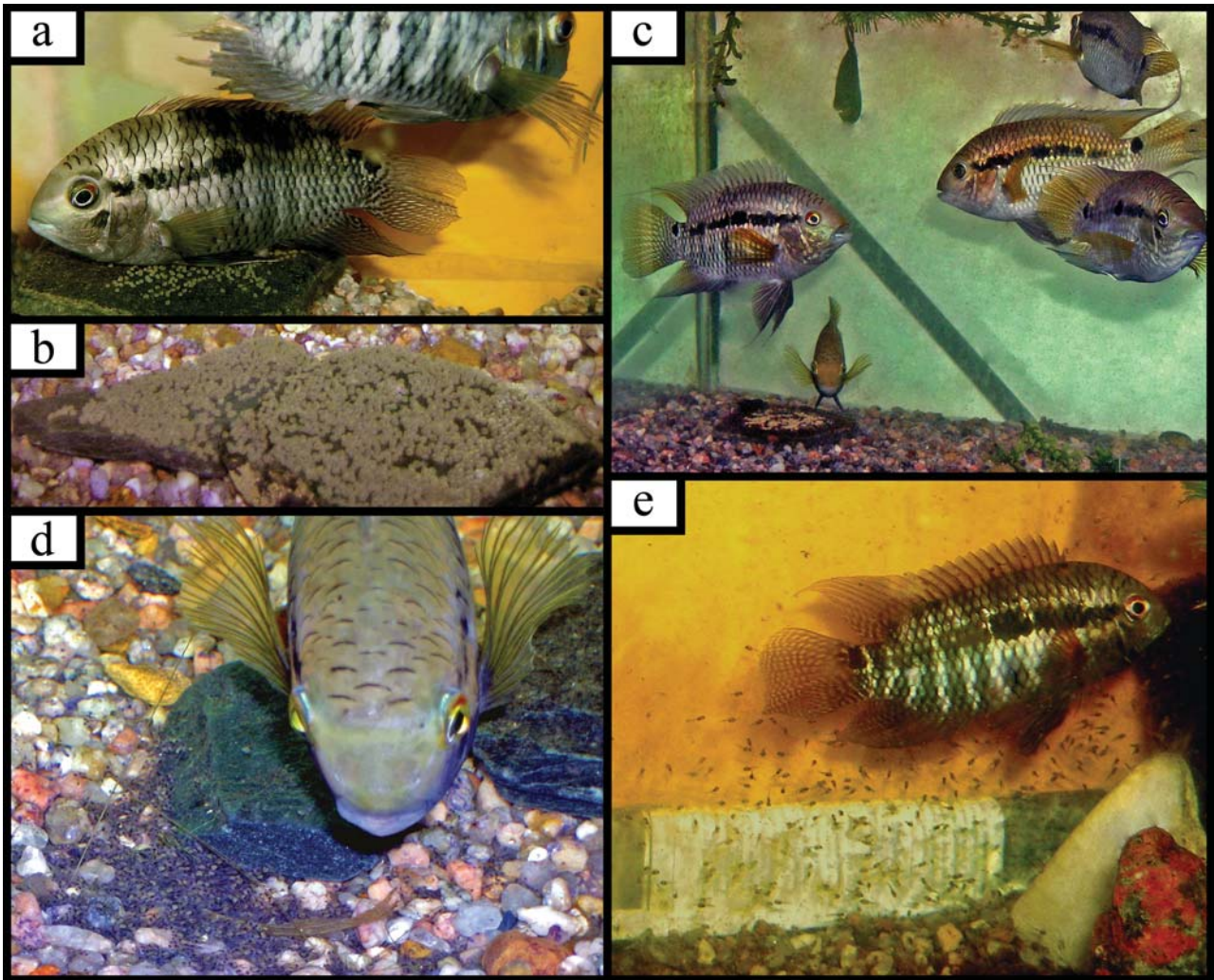


Fig. 4. Reproductive phases: **a)** Female laying eggs while male waits to fertilize them. This process is then repeated; **b)** Spawn on a flat dark stone. Generally between 400 = 800 eggs are laid, depending on female size; **c)** Female guarding the eggs while male attacks an intruder male. Generally one of the parents remains guarding the fry while the other feeds or defends the territory; **d)** Female fanning non swimming fry after transferring them to a previously dug pit; fry hatched 3 days after fertilization; **e)** Male guarding free swimming fry (8 days after fertilization); parents seem to attract the fry by shaking its dark pectoral fin.

limited by the available spawning surface. After spawning, both parents took care of the eggs by defending them from other fish and animals, attacking any intruder into their territory. Both alternatively fanned the eggs by beating their pectoral fin over the clutch. When one parent left the clutch for food or to attack an intruder into their territory, the other generally stayed taking care of the eggs or larvae. In consequence it was very unusual to observe fry without one of the parents taking care of them (Fig. 4c). Dead eggs, which look white and usually are attacked by fungi, are removed by the parents. Parents will start to dig in the gravel making nests of about 10-20 cm of diameter and 5-10 cm depth (Fig. 4d). Larvae started to hatch at the beginning of the third day at a water temperature of 25°C, and were subsequently transferred by the pair to a previously dug pit. During five more days larvae remained attached to the bottom of the pit by a head adhesive-gland and both parents took care of them and transferred them to different pits. Larvae started swimming 8 days post fertilization (Fig. 4e). During this period the reproductive pair (RTm and RTf) expanded their territory size and increased their aggressiveness toward non-dominant individuals. They continually attacked the other fish during the whole period of fry care (Fig. 5). Larvae followed their parents foraging on the ground. Both parents attracted them by shaking their pelvic fin that showed a dark grey color at this stage. This behavior was especially observed when there

was a perturbation near the aquarium. As described herein, the same dark color was also observed in the trunk and head ventral region in both parents. Animals were sacrificed at this stage.

Male gonadosomatic indexes

Gonadosomatic indexes did not show significant differences between Reproductive ($0,18 \pm 0,047$) and non Reproductive males ($0,10 \pm 0,015$).

Plasma cortisol levels

Non Reproductive males and females (nR-nT) had significantly higher cortisol levels than Reproductive males and females respectively. Also, males showed higher cortisol levels than females (Fig. 6).

Pituitary hormone content

The relative optical density of ir- β -FSH, β -LH, and SL bands (normalized to α -tubuline) was measured in order to test the hypothesis that the social status affects the pituitary content of these hormones. The analysis showed that R males had significantly higher pituitary content of β -FSH and SL than nR males (Fig. 7a), while no significant differences were detected in β -LH pituitary content. In females, no significant differences were found in the pituitary content for any measured hormone (Fig. 7b).



Fig. 5. Reproductive territorial male attacking a non reproductive territorial male.

Discussion

We described for the first time *C. dimerus* social and reproductive behavior in the laboratory, including the establishment of dominance hierarchy. We also characterized the different social statuses and body coloration patterns associated with them.

During the cold, short-photoperiod season, *C. dimerus* do not reproduce and shows the gregarious behavior and color pattern described in the results section. In this species, short photoperiod reduces male aggression either related to territory selection or its defense, and also females under this photoperiod are never chosen as mates by the males (Fiszbein *et al.*, 2010). This indicates that short photoperiod inhibits reproductive behavior and, in consequence, diminishes aggressiveness. Social hierarchies are not maintained during this season and almost no aggressive interactions are observed (Alonso, pers. observ.).

The start of the warm, long-photoperiod season is followed by the social interactions that determine the social hierarchy among the individuals of this species. Size is the most important factor in the establishment of social hierarchy, probably being related to strength, which is supported by the observation that hierarchies are established by aggressive interactions, including physical contact. All threatening displays have the common effect to make the fish seem “as larger as possible” by maximizing the visible area. This could be related to size, which in turn may be a good indicator of strength and would explain why in our experiments the most

dominant male, which is reproductively active, was always the largest male in the aquarium. In the convict cichlid, *Amatitlania nigrofasciata*, females show no preference based on male reproductive experience or dominance status, but prefer the largest between two males (Noonan *et al.*, 1983; Gagliardi-Seeley *et al.*, 2009). Male convict cichlids have been shown to prefer gravid over non gravid females, and to prefer the largest between two gravid females (Nuttall & Keenleyside, 1993). In free-ranging fish, it was observed that breeding fish paired up according to size; large males with large females and small males with small females. Small males and, in some cases, small females were excluded from breeding (Wisenden, 1995). An outstanding feature of *C. dimerus* behavior is that

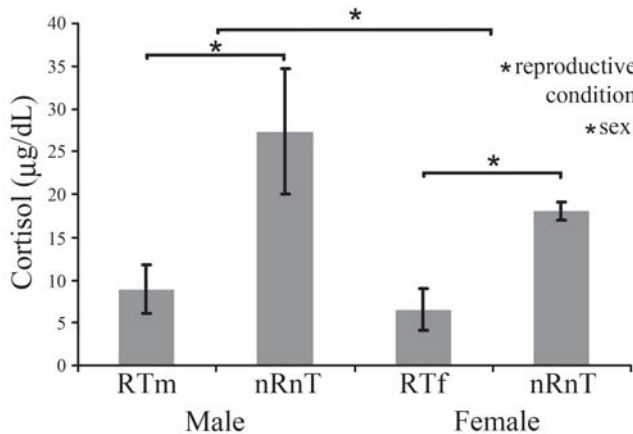


Fig. 6. Plasma cortisol levels in reproductive territorial (RT) and non reproductive non territorial (nRnT) males and females (N=4). Cortisol levels were higher in males than females. Non Reproductive individuals presented higher levels than reproductive ones for both sexes ($p < 0.05$). No significant interaction was detected between sex and reproductive state. A three factor ANOVA was performed to analyse this data, with experiment repetition as a random factor. Asterisks indicate a statistical difference at $\alpha = 0.05$ for the factor sex (male vs. female) and reproductive condition (reproductive vs. non reproductive).

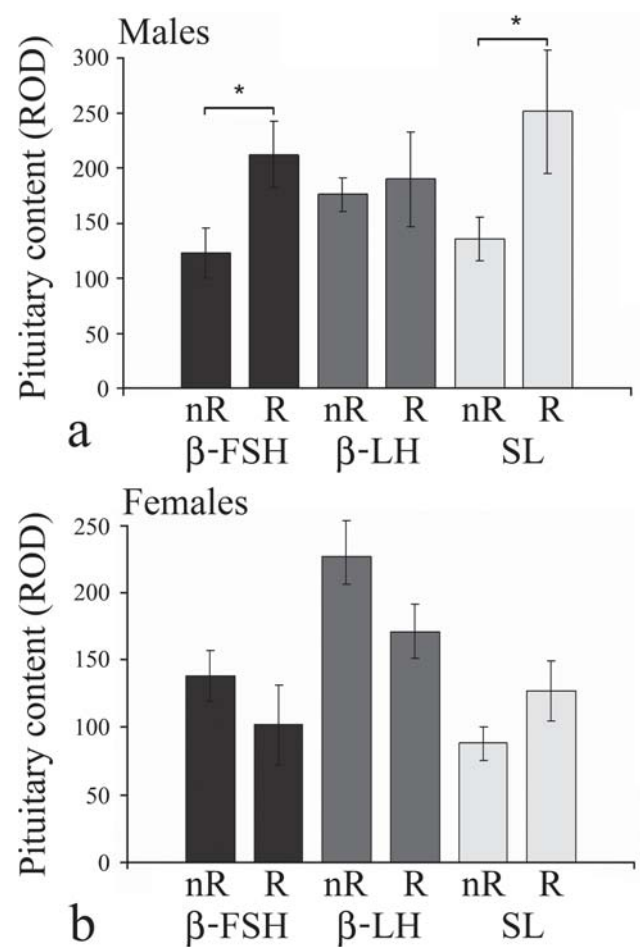


Fig. 7. Semiquantitative analysis of pituitary hormone content from reproductive (R) vs. non reproductive (nR) individuals (n = 4). Values are expressed in arbitrary units (a.u.) as means \pm SEM and normalized to α -tubuline. ROD = relative optical density. Asterisks indicate a statistical difference at $\alpha = 0.05$ with a Mann-Whitney test. **a)** Males: Greater pituitary β -FSH and SL content was observed in R males compared to nR males ($p < 0.05$). No significant differences were observed in β -LH pituitary content between R and NR males. **b)** Females: No significant differences were observed in β -LH, β -FSH, and SL pituitary content between R and nR females.

the threatening displays were the same displays performed towards females accepted as potential mates by the male. These intra-sexually selected behavioral features were probably reinforced by inter-sexual selection (reviewed in Anderson, 1994) because these are good predictors of male size and selecting these features would positively correlate with a better fitness for females. This would also account for males being larger than females, given that this feature may have evolved through inter and intra-sexual selection.

As observed in *C. dimerus*, convict cichlid females, despite selecting one male over another male early in the courtship period, continue to visit both males until spawning. It was suggested that this assessment behavior would more closely resemble a bet-hedging tactic rather than female's indecision. Higher aggressiveness in males than females during the prespawning period was also observed, and females invested more time than males on parental care (Gagliardi-Seeley *et al.*, 2009). As in *Amphilophus citrinellus* (Rogers, 1987), *C. dimerus* males showed more aggressiveness toward other males than toward females.

In *C. dimerus* those individuals that acquire reproductive territories adapt their physiology to reproduction and show the bright background body color pattern, as opposed to the dark color pattern of nRnT, which is probably a submissive display. In the convict cichlid, sexual dichromatism was shown to affect intrasexual aggression among females (Beeching *et al.*, 1998). Those fish lower in the social hierarchy that not obtained reproductive territories searched for refuge under the leaves of plants and aggressively defended this position from subordinate individuals in the social hierarchy. The maintenance of the dominance hierarchy among non-reproductive individuals may be related not only to access to resources such as food (*i.e.*, Arthropoda living in between plants) and refuge, but also may eventually determine access to new available reproductive territories due to death of reproductive individuals, or environmental changes such as flooding of nearby areas, for example.

The fast changes in body color pattern of these fish allow them to have a complex and effective signaling system capable of sending complex information quickly and reinforcing that visual cues are very important in fish communication. This feature of their visual communication may have a positive adaptive value by permitting a faster adaptation to changes in dominance status and life in a variable environment. Changes in the dark stripes, lines and body blotches were quick, suggesting that these changes were probably mainly controlled by nervous system, although background coloration changes are long term adaptations and so are more likely to have mainly an endocrine control.

Similarities between behaviors of different Neotropical cichlids may indicate that some of these behavioral patterns were well conserved during the evolution of this lineage or converged due to similar selective pressures related to life in similar environments. However, the former hypothesis may be the more parsimonious because there are many other similar features among these fishes such as color pattern (*i.e.*, bright

in reproductive dominant individuals, dark stripes). For a better comprehension of the evolution of behavior and reproductive physiology in cichlids phylogenetic studies that include independent comparisons should be done.

In the African cichlid *Astatotilapia burtoni*, (= *Haplochromis burtoni*) territorial males develop their coloration patterns faster, weigh more and have larger and more developed gonads than non-territorial males (Fernald, 2002). In established groups of the highly territorial group-living African cichlid *Neolamprologus pulcher*, dominant individuals have higher androgen levels and greater relative gonad investment than the subordinates ones (Fitzpatrick *et al.*, 2006; Desjardins *et al.*, 2008) but plasma androgen levels did not correlate with relative gonad investment in females or males, winners or losers after territory competition (Taves *et al.*, 2009). Considering the experimental design of the present work, in *C. dimerus* no differences were found between GSIs of RT and nRnT males. This could be related to the short-term nature of the study that probably precluded any difference related to social status and gonadal investment. Also, the fact that dominant males were sacrificed 8 days post-spawning/spermiation may have resulted in a low GSI values for these individuals. Since the reproductive opportunities may occur at any time during the reproductive period, it would have a positive adaptive value for nR individuals to always be ready for reproduction during this period.

The fact that different cortisol levels are related to social status in *C. dimerus* indicates that social statuses represent different levels of stress. In both sexes, nRnT individuals had higher serum cortisol than RT ones. In *A. burtoni* it was clearly demonstrated that nT males had more plasma cortisol levels than T ones, but this is also highly dependent on community conditions and length of time in these conditions (Fox *et al.*, 1997; White & Fernald, 1997). In *C. dimerus* cortisol may serve as an endogenous signal relating the social environment to the social status of the animal, behavior and internal reproductive state. Physical contact aggressions from the R individuals towards nR ones may inhibit the signals that trigger reproduction by increasing cortisol levels, excluding therefore these individuals from competition and leaving more resources available for the reproductive pair and their fry. Submissive displays may be more likely to occur in fish with high cortisol levels, with the positive adaptive value of diminishing attacks by dominant individuals. The higher cortisol plasma levels observed in males when compared to females may be due to males showing greater aggressiveness, which was qualitatively observed. Experimental manipulation of serum cortisol levels and behavioral experiments should be performed to address these issues.

In *C. dimerus* the lower levels of β -FSH pituitary content in nR males, compared to R ones, may indicate an inhibition of β -FSH synthesis and/or secretion. This could be due to an inhibition, via plasma cortisol, of GnRH-1 synthesis or release. However, we could not rule out an effect on the onset of spermatogenesis in RT males considering that they were sampled 8 days after spermiation. The higher levels of SL

pituitary content in RT males could be related to body background color changes related to territory defense, reproduction and parental care. It was previously seen in this species that animals adapted to black background showed a darker background body color and higher SL pituitary content than animals adapted to white background (Cánepa *et al.*, 2006). Also, differences found in SL related to the reproductive state of females of the pejerrey *Odonesthes bonariensis* (Vissio *et al.*, 2002) could be related to differences in body color rather than a direct influence on the reproductive axis. No significant differences in β -FSH, β -LH, and SL pituitary content between RT female and nRnT females were found.

Further studies will be necessary to understand the factors that affect the establishment of social hierarchies and the dynamics of social status. In order to achieve a better understanding of the reproductive system of this species, future studies should focus on the physiological mechanisms underlying the reproductive inhibition due to high cortisol levels of the individuals lower in the social hierarchy. Also sexual selection experiments, together with field observations on the natural habitat of *C. dimerus* should be performed.

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