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A multidisciplinary study on social status and the relationship between inter-individual variation in hormone levels and agonistic behavior in a Neotropical cichlid fish

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ABSTRACT

Social animals with hierarchal dominance systems are particularly susceptible to their social environment. There, interactions with conspecifics and hierarchal position can greatly affect an individual's behavior, physiology and reproductive success. Our experimental model, Cichlasoma dimerus, is a serially-monogamous Neotropical cichlid fish with a hierarchical social system, established and sustained through agonistic interactions. In this work, we aimed to describe C. dimerus social structure and its association with hormonal profiles and testicular cellular composition. We recorded and quantified agonistic interactions from the territorial pair, i.e. the top ranked male and female, and the lowest ranked male of stable social groups. Plasma levels of 11-ketotestosterone (11-KT), testosterone, 17β-estradiol (E_2) and cortisol were measured by ELISA. Results show that territorial pairs cooperatively guarded the territory, but rarely attacked in synchrony. Territorial males had higher testosterone and 11-KT plasma levels than non-territorial males, while E_2 and an index of its metabolization from testosterone were higher in non-territorial males. No difference was observed in cortisol levels. Plasma 11-KT and an index of the conversion of testosterone to 11-KT, positively correlated with the frequency of aggressiveness, while E_2 showed the opposite pattern. Territorial males had a higher gonadosomatic index than non-territorial males. The quantification of testicular cellular types revealed that the percentage of spermatocytes and spermatids was higher in non-territorial males, while territorial males showed a greater percentage of spermatozoa. Thus, C. dimerus male social position within a stable hierarchy is associated with distinct behaviors, steroid levels and testicular degree of development.

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Introduction

An animal's social environment can have profound effects on its behavior and physiology (Bernhardt et al., 1998; Chu and Wilczynski, 2001; Galhardo and Oliveira, 2014; Lacava et al., 2011; Mazuc et al., 2003; Pinto et al., 2011; Sessa et al., 2013). Yet, it is not all up to external factors, as individual's internal environment (i.e. physiological and motivational states) also contributes to determine its behavioral output (Eisenegger et al., 2010; Quiring, 1944; Soma, 2006; Tudorache et al., 2013). These intertwined and usually bidirectional effects are of particular importance in social species in which hierarchal dominances are established. In such cases, social status can greatly affect an individual's behavior and physiology through interactions with its conspecifics (Sapolsky, 2005).

The physiological basis of social dominance is strongly linked to sex steroids and glucocorticoids, as individuals of distinct social status (i.e. dominant vs. subordinate) usually differ in their hormone profiles. Dominant animals are frequently aggressive and show elevated androgen levels (Albert et al., 1986; Duckworth et al., 2004; Oliveira et al., 2002; Renn et al., 2012; Taves et al., 2009), whereas subordinates perform submissive behaviors, often accompanied by elevated glucocorticoid concentrations (Alonso et al., 2011; Greenberg et al., 1984; Morandini et al., 2014; Sherman et al., 2012). Even though estrogens have been historically associated with female-typical behavior, evidence has been accumulating on its active role in the regulation of male aggressive behavior (Huffman et al., 2013; Soma et al., 2000b;

Schlinger and Callard, 1990). Nonetheless, acquired position within a social hierarchy not only affects (and is affected by) the endocrine landscape, but also other aspects of physiology, including spermatogenesis (Maruska and Fernald, 2011), sperm maturation (Koyama and Kamimura, 1998), oogenesis (Filby et al., 2010), gene expression (Kroes et al., 2006; Runcie et al., 2013) and neurogenesis (Kozorovitskiy and Gould, 2004; Maruska et al., 2012).

The integration of the external and internal environment determines an organism's behavioral output. Individual variation in this output has been interpreted for many years as noise around an average, but in the last decades interest has grown in the physiological bases of interindividual variation in social behavior (Bennett, 1987; Crews, 1998; Knussmann et al., 1986; Maestripieri et al., 2009; Schlinger and Callard, 1989). By analyzing the "noise" into its individual components, it is possible to predict and better understand an individual's performance. For example, individual variation in baseline cortisol levels positively correlated with post-stress cortisol in dwarf hamsters (Guimont and Wynne-Edwards, 2006), and individual differences in spontaneous brain cortical activity correlated with individual differences in performing novel perceptual tasks in humans (Baldassarre et al., 2012). Of particular attention have been hormone–behavior interrelationships (i.e. the correlation between hormone concentration and the intensity or frequency of the hormonal regulated behavior), and more recently, with the advance in molecular biology, integrative analysis of behavior, hormones and target organ receptivity and response (see Ball and Balthazart, 2008 for review). However, even in well established "cause"–effect hormone–behavior relations, many studies have not found an association between individual variation in hormone concentration and behavior (e.g.: testosterone and male aggression, Arlet et al., 2011; Vullioud et al., 2013; testosterone and male sexual behavior, Balthazart et al., 1977; Feder, 1984), highlighting its complexity.

In the last decades, cichlid fish have emerged as pivotal models for ethological studies due to their great variety of social systems (Baerends and Baerends-van Roon, 1950; Barlow, 2000; Sefc, 2011), easy maintenance and readily behavioral observation under laboratory conditions. *Cichlasoma dimerus* (Heckel, 1840), locally known as *chanchita* in Argentina, Bolivia, Paraguay and Uruguay, is a seriallymonogamous Neotropical cichlid with a hierarchical social system established and sustained through agonistic interactions (see Pandolfi et al., 2009 and Ramallo et al., 2014 for review). This highly social fish offers an ideal opportunity for the study of hormone–behavior relationships within a rich social context. *Chanchitas* are found in one of two basic and interchangeable social phenotypes that are linked to social and reproductive status. Non-territorial (NT) *chanchitas* have dark background body coloration and are socially denied immediate access to reproduction by the brightly colored territorial (T) conspecifics. Male–female pairs occupying top ranked positions aggressively guard a territory, an asset that is critical for reproduction in this species, as it provides a substrate where they will spawn and later begin with bi-parental care of the eggs and larvae. While the joint defense of an area by the territorial pair (pair cooperative territoriality) is among the most common reproductive strategy of Neotropical cichlids (Keenleyside, 1991), only few studies have analyzed it on a quantitative level (Mackereth and Keenleyside, 1993; Rogers, 1988; Teresa and Goncalves-de-Freitas, 2011).

In this work we aimed to (1) expand our knowledge on *C. dimerus* social structure with emphasis on the analysis of the less studied pair cooperative territoriality and (2) investigate the reciprocal relationships between social status, agonistic behavior, and physiology. In particular we hypothesized that individual variation in agonistic behavior is associated with individual variation in sex steroid and stress hormones. We predicted that androgens would be associated with a higher frequency of aggressive behavior, while we expected a correlation between estradiol, cortisol and submissive behavior. We began by quantifying the frequency of distinct types of agonistic interactions performed by the T pair and the lowest ranked NT fish, and employed them for the construction of an index representative of the overall agonistic level. We then evaluated the relationship of this index with different physiological parameters. Unlike most studies in which dominance relationships are evaluated in dyadic encounters (Fiszbein et al., 2010; Pinho-Neto et al., 2014; Oliveira et al., 2002; Tubert et al., 2012), this work analyzes the physiological aspects of social relationships within the context of a social group, which represents a more natural setting for this species.

Material and methods

Animals

We obtained male and female adult specimens of *C. dimerus* from wild populations captured in Esteros del Riachuelo (27°35′S; 58°45′W; Corrientes, Argentina), using fishing nets. Fish were then transferred to our laboratory in Buenos Aires and housed in community tanks (150 l, 8–10 fish per tank) with artificial aquarium plants and stones, under conditions mimicking their natural reproductive habitat: 25 ± 2 °C and 14:10 light:dark cycle (Casciotta et al., 2002) with full spectrum illumination. Every morning, we fed fish ad libitum with fish food sticks (Koi Vibrance Color Enhancer Fish Food — Tetra Brand). The animals were allowed to acclimate to aquaria conditions for at least three weeks before their incorporation into the experimental set up.

Under all circumstances we took appropriate actions 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 institutional (Comisión Institucional para el Cuidado y Uso de Animales de Laboratorio, Facultad de Ciencias Exactas y Naturales, Universidad de Buenos Aires) and national (Comité Nacional de Ética en la Ciencia y la Tecnología) regulations. Procedures were in compliance with the Guide for Care and Use of Laboratory Animals (National Research Council, 2011).

Experimental design

To obtain territorial and non-territorial individuals, we placed six size-matched fish in an experimental tank (53 l; temperature 25 ± 2 °C and 14:10 light:dark cycle with full spectrum illumination; n = 9). A layer of gravel (~2 cm) covered the bottom of the aquaria where artificial aquarium plants and stones were anchored, along with flat slabs where this species tends to lay its eggs. Members of each group were assigned based on their overall similar size, as relative body size is a major determinant of the outcome of agonistic interactions in this species (Alonso et al., 2011). Briefly, we weighed and measured standard and total length of all available fish at the beginning of each experiment. We then incorporated the morphological variables into a K-means clustering analysis (number of iterations = 10), and the six members of each cluster with the shortest Euclidean distances were selected as constituents of an experimental group. As *C. dimerus* does not show any obvious sexual dimorphism, each group had a random sex ratio.

Once the six size-matched fish were introduced into the experimental arena, we allowed animals to acclimate for 24 h. On the following days we proceeded with the visual identification of individual fish based on distinct marks on their bodies and fins, and evaluated agonistic interactions for 15 min twice daily, once in the morning and again in the afternoon. By observing the relationship of single fish with others (number of interactions, wins and losses, defined as the performance of aggressive actions by the winner that resulted in submissive behavior by the loser), we determined the position of each fish within the established linear hierarchy (Fig. 1), and it also allowed us to perform a qualitative analysis of the dynamics of hierarchy development.

The territorial pair in each group was established in approximately a week. The pair was identified as the two top ranked individuals that were not aggressive with each other, but together guarded a territory
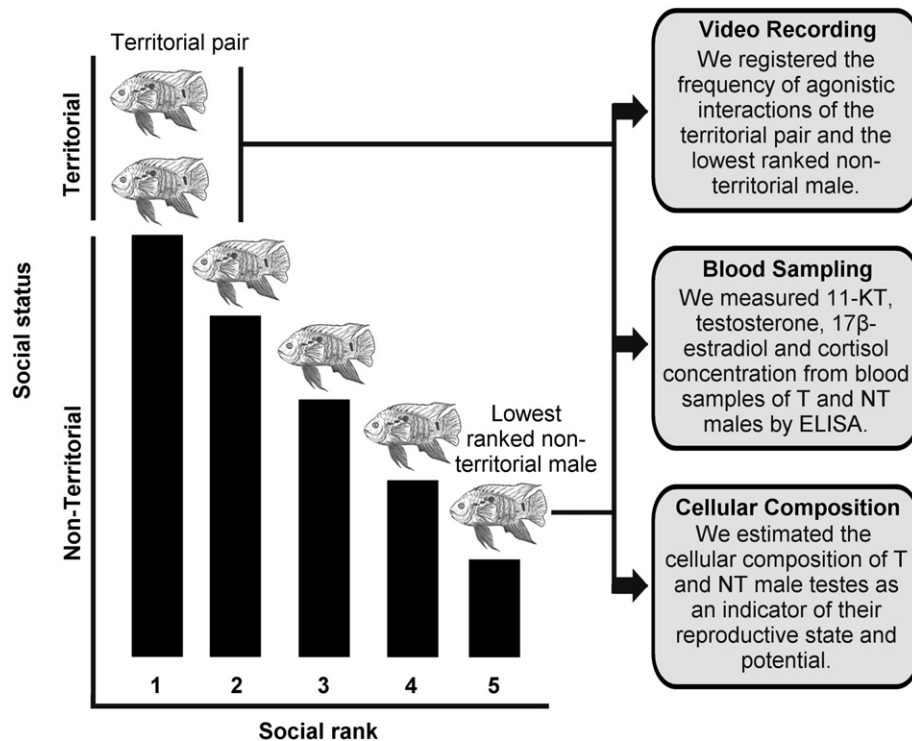
Fig. 1. Diagram summarizing the general experimental design. Experimental aquaria housed 6 size-matched fish (random sex ratio). Once the linear hierarchy was established, top and bottom ranked individuals were identified and analyzed. Recorded agonistic interactions include chases, bites, approaches, escapes and passive coping. For the study of the testes we estimated the percentage of type A and B spermatogonia, spermatocytes, spermatids, spermatozoa and interstitial tissue. Abbreviations: T, territorial; NT, lowest ranked non-territorial; 11-KT, 11-ketotestosterone.

from the other fish in the aquarium, and began performing reproductive displays (7.11 ± 1.42 days until the observation of pre-spawning behavior; minimum: 3 days; maximum: 14 days). The latter includes body shaking, rubbing of the body and genital papillae on the spawning site, stone nipping and pit digging by both members of the pair (Alonso et al., 2011). The morning when such behaviors were first detected, we video recorded the aquarium for the qualitative and quantitative analysis of intra-sexual agonistic interactions and pair dynamics. Filming was performed between 11:00 and 13:00 h and average recording time was 23.4 min (minimum: 19.2 min; max: 23.7 min).

After filming, we removed the territorial pair and the lowest ranked fish from the aquaria, and drew blood by puncture of the caudal vein with heparin-coated syringes (needle: 27 gauge × 1/2 in.) for the measurement of steroid hormones. To minimize possible effects of circadian variations, all samples were collected in heparin-coated tubes between 14:00 and 14:30 h. We kept blood overnight at 4 °C and later centrifuged it at 3000 rpm for 15 min, withdrew the plasma and stored it at −20 °C until assayed. As there is a temporal gap between the recording of agonistic behavior and blood drawing, the possibility exists for a disconnection between recorded performance and hormonal levels due to circadian rhythms and varying interactions with different group members. Thus, in a distinct set of experimental groups (n = 4) we recorded the frequency of agonistic interactions of focal fish between 11:00 and 13:00 h, and again between 14:00 and 14:30 h, calculated the index of agonistic behavior (see below for details) and analyzed its repeatability (R) following the guidelines by Nakagawa and Schielzeth (2010). This index, R, is a measure of behavioral consistency (Lessells and Boag, 1987), which ranges from zero (no consistency) to one (highly consistent). The overall frequency of agonistic behavior was highly consistent (R = 0.99; 95% CI: 0.96–1.01; p < 0.0001) between the two evaluated time points. This implies that if we had quantified agonistic behavior immediately previous to blood drawing (between 14:00 and 14:30 h),

we would have obtained statistically the same indexes of agonistic behavior as we did by measuring behavior earlier.

In all cases, fish were then immediately anesthetized with 0.1% benzocaine until opercula movement ceased, and the weight and total and standard length of each animal were recorded. We next euthanized them by decapitation. We determined the sex of the fish by gonadal inspection and dissected and weighed the gonads of T and NT fish for the calculation of their gonadosomatic indexes (GSI; [gonad weight / total body weight] × 100). Next we proceeded with fixation in Bouin's solution of one of the gonads (right or left; randomly selected) per fish, to evaluate its cellular composition as an indicator of its reproductive state and activity.

As we intended to study *C. dimerus* behavioral and hormonal profile related to social status (territorial vs. non-territorial), the sex of the non-territorial individual determined the nature of these comparisons, as we focused on intra-sexual variation. In this work we only present data from T and NT males (n = 9).

Video analysis

Social status: T vs. NT males

We recorded the number of all agonistic interactions performed by the T and the lowest ranked NT male. These are all social encounters where T and NT males were involved, whether between them or with the other fish in the aquarium. Aggressive displays included chases (when the focal fish swam behind a fish swimming away from it for a relative long distance), bites (performed by the focal fish on the fins or sides of another fish) and approaches (short distance and quick chasings that did not end in physical contact). In this paper we use the term "attack" in reference to any of the abovementioned aggressive displays. Submissive behavior included escapes (when the focal fish swam away from other fish chasing, biting or approaching it) and passive coping

(when the focal fish did not escape or moved when bitten or approached while almost in a vertical position, head upwards). As recording times varied slightly between experiments, agonistic interactions are presented as the frequency of a given behavior per minute.

To study the relationship between agonistic behavior and hormone profiles, individual behavioral frequencies were analyzed with a Principal Component Analysis (PCA), and the resulting scores of each fish for the first principal component (PC1) were used as an index of overall agonistic behavior. A high positive score for a given fish is associated with an elevated frequency of aggressive behaviors and a low frequency of submissive behaviors, whereas the opposite occurs for more negative scores. Non-territorial and territorial males are clearly separated on PC1 in a bivariate scatter plot of the first and second principal components (Fig. 2).

Pair dynamics

For the description of the territorial pair's behavior, we quantified the frequency of T female agonistic interactions in the same manner as described for males. To analyze pair dynamics, that is whether T male and female acted jointly or on an interleaved pattern, we performed a one-zero sampling every 30 s on video playbacks, where we recorded the occurrence of territorial aggression (1: chasings, approaches or bites; 0: no aggressive behavior) and the identity of the aggressor (i.e. T male, T female or both). The proportion of all sample intervals where aggressive behavior was scored, was positively correlated with the frequency of aggression under continuous recording (p = 0.0003; Pearson's correlation coefficient = 0.78, r^2 = 0.62; males and females combined). Thus, this sample interval is a good indicator of overall aggressive behavior frequency.

Hormone analyses

We measured 11-ketotestosterone (11-KT), testosterone, 17β-estradiol (E_2) and cortisol from plasma samples of T and NT males using commercial ELISA kits. As cortisol may quickly increase due to fish manipulation (Fox et al., 1997), we quantified cortisol from blood samples which drawing time was less than 4 min after netting. Working dilutions were 1:6 for 11-KT (some T males samples had to be diluted even further, up to 1:50, as 11-KT concentration was over the upper curve limits), 1:2 for testosterone and E_2 and undiluted for cortisol. In all cases samples were assayed in duplicate and analyses were carried on samples in which the coefficients of variation were below 20%, following the manufacturer's instructions. Some samples did not have enough volume for the appropriate measurement of all four hormones, thus some analyses have a lower number of analyzed samples (n < 9).

Intra-assay variation was 11.1% for 11-KT (detection limit: approximately 1.3 pg/ml; Cayman Chemical Company, MI, USA), 10.56% for testosterone (detection limit: approximately 0.07 ng/ml; IBL International, Hamburg, Germany), 18.58% for E_2 (detection limit: approximately 9.714 pg/ml; IBL International, Hamburg, Germany) and 8.56% for Cortisol (detection limit: approximately 2.46 ng/ml; IBL International, Hamburg, Germany), while inter-assay variation was 5.40%, 15.58%, 5.30% and 10.28% respectively. Parallelism to standard curves was assessed by measuring hormone concentration in serially diluted samples (4 different dilutions). Correlation coefficients were 0.992 for 11-KT, 0.990 for testosterone, 0.983 for E_2 and 0.972 for cortisol after log transformation.

Quantification of testicular cell types

Testes were fixed by immersion in Bouin's solution for 24 h at room temperature, dehydrated through an ascending series of ethanol, clarified with xylene and embedded in paraplast. The central region of each testis was then serially sectioned at 5 μm in the transverse plane, mounted on gelatin-coated slides and stained with Masson Trichrome (hematoxylin, acid fucsin-xylidineponceau, phosphomolybdic acid and aniline blue). Three randomly chosen sections from each T and NT fish were then examined with a Microphot FX (Nikon) microscope at 400× and digitally photographed (Coolpix 4500, Nikon).

In order to quantify the testicular cell types, 60 randomly generated points were overlaid on each photomicrograph using CPCe software, and the cell type beneath each cross-hair point was identified. In a pilot study, 60 points proved sufficient to adequately determine the percentage of all cell types (we tested 45, 60, 75 and 90 points; p > 0.05 for 60, 75 and 90). We followed the detailed histological description of *C. dimerus* testis by Rey Vázquez et al. (2012) to correctly identify each cell type. Thereafter we calculated the percentage of type A and B spermatogonia, spermatocytes (primary and secondary combined), spermatids, spermatozoa and interstitial tissue. Percentages were then averaged from the three photomicrographs for each cell type.

Data analyses

All statistical analyses were performed using Statistica 8 (StatSoft®). We used the non-parametric Wilcoxon Signed-Rank test for paired samples to analyze the variation in the frequency of submissive and aggressive behavior between T and NT males and T male and female, as data did not meet parametric test assumptions. Effect sizes were calculated according to r = $\frac{z}{\sqrt{N}}$, where z is the test statistic and N is the total number of samples. In addition, we performed a paired T-Test to analyze whether the occurrence of a synchronous attack by the territorial pair differed of that expected by chance. For this we compared the product of the frequency of male territorial aggression to female territorial aggression (i.e. the likelihood of the pair attacking simultaneously), with the actual observed frequency of concurrent attacks. Effect size was calculated using Cohen's d.

Variations in hormonal profiles and GSI associated with social status, were analyzed by means of a Randomized Block Design Analysis of Variance (RBD-ANOVA), considering each aquarium as a block. To avoid the appearance of false positives as a result of multiple hypotheses testing within each of the abovementioned tests (i.e. hormone profiles and inter- and intra-sexual behavioral analysis), we applied the false discovery rate (FDR) two-stage sharpened method correction (Benjamini et al., 2006), and all presented p-values are modified accordingly using the spreadsheet-based software provided in Pike (2011). Effect sizes were estimated using general Eta-squared, η^2_G.
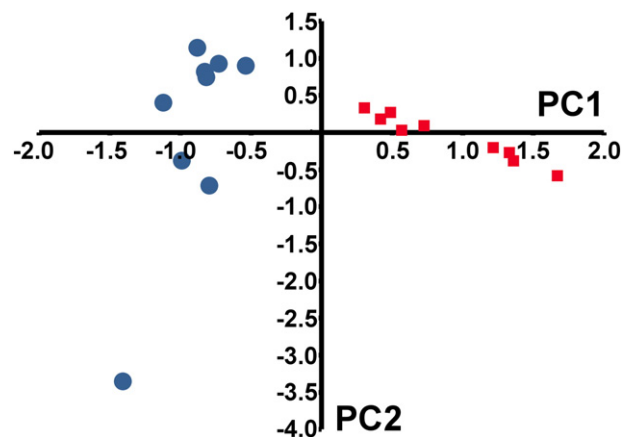

Fig. 2. Bivariate scatter plot of scores of territorial (red squares) and lowest ranked non-territorial males (blue dots), resulting from a principal component analysis. The analysis incorporated the frequency of all five agonistic interactions recorded. Abbreviations: PC1, first principal component; PC2, second principal component.

We used Pearson's c correlation coefficient to study the relation between the index of agonistic behavior (PC1) and hormone concentrations. To account for multiplicity, p-values were also corrected by FDR.

For the study of the cellular composition of the testes of T and NT males we performed various RBD-ANOVAs, one for each cell type, and p-values were corrected by FDR. Effect sizes were calculated using general Eta-squared.

Finally, all data sets were checked for normality and homoscedasticity and, if needed, were square root-, cos- or log-transformed. In some cases we found data to be outliers (studentized residuals $> \pm 2$), and they were excluded from the analyses. The data are presented as mean $\pm$ standard error of the mean (SEM) and we set statistical significance at $p < 0.05$.

Results

Daily observations of the experimental aquaria revealed that between 24 and 48 h after fish were first placed within the experimental tank, the presence of a T fish was readily detectable. This T fish defended a territory and aggressively attacked all other *chanchitas* within the aquarium. During hierarchy establishment, T fish were occasionally challenged by others, mostly through threatening displays such as fin erection and opercula opening, or even physical interactions like mouth holding. In eight out of nine experimental aquaria, this early detected T fish continued to dominate and later formed a territorial pair, usually with the second most dominant fish. In seven out of nine cases, firstly detected T fish were males.

Social status: T vs. NT males

Once the pair engaged in pre-spawning behavior, more than 50% of their agonistic repertoire consisted of bites directed to NT fish (Fig. 3a). On the other hand, the lowest ranked NT males primarily escaped from the attacks of dominant fish in the aquaria and seldom behaved aggressively (Fig. 3b).

As expected, T males performed significantly more chases ($z = -2.67$, $r = 0.63$, $p = 0.01$; Fig. 4a.i), bites ($z = -2.67$, $r = 0.63$, $p = 0.01$; Fig. 4b.i) and approaches ($z = -2.67$, $r = 0.63$, $p = 0.01$; Fig. 4c.i) than NT males. Nonetheless, one NT male also attacked other fish, usually the one immediately previous it in the linear hierarchy, as a response to an immediately previous attack. Sometimes territorial fish were also attacked by subordinates, although we only observed this five times out of the 1547 recorded agonistic behaviors. In such cases, T males responded by moving away from the aggressor (i.e. escape; 4 out of 5 times) or by coping passively (1 of 5 times), behaviors more frequently performed by NT males (Passive coping: $z = -2.38$, $r = 0.56$, $p = 0.015$; Fig. 4d.i; escapes: $z = -2.67$, $r = 0.63$, $p = 0.01$; Fig. 4e.i).

Territorial pair dynamics

The average frequency of aggressiveness of T females did not differ from that of T males (2.7 ± 0.3 vs. 3.6 ± 0.6, females vs. males; $F_{1,7} = 5.09$, $\eta^2_G = 0.23$, $p = 0.06$). However, T females performed almost 50% fewer chases ($z = -2.10$, $r = 0.50$, $p = 0.027$; Fig. 4a.ii) and approaches ($z = -2.38$, $r = 0.84$, $p = 0.015$; Fig. 4c.ii) than T males.

The territorial pair seemed to act on an interleaving pattern (Fig. 5a) and rarely attacked together ($F_{1,14} = 24.45$, $\eta^2_G = 0.63$, $p < 0.01$). The chance of simultaneous attack met that expected by mere chance ($T_7 = 0.04$, Cohen's $d = 0.01$, $p = 0.97$; Fig. 5b). Usually, when one member of the pair attacked subordinate fish, the other remained close to the pairs' territory foraging for food, resting, or rubbing its body and genital papillae on the spawning site, stone biting or pit digging. On occasions, when either the T male or female was threatened by other fish, the other member of the pair intervened in the dyad by also attacking the threatening animal.

When considering the occurrence of aggressive behavior by the territorial pair all together (i.e. male and female combined) we found that aggression is more likely to occur in trains of successive attacks, separated by periods of resting, rather than occurring in a continuous or random manner (Fig. 5c). Even though we only registered attacks every 30 s in a 1–0 fashion, the proportion of all sample intervals where aggressive behavior was recorded is a good indicator of the frequency of aggressiveness under continuous sampling. Thus, a follow-up of sample intervals where attacks were scored, most likely reflects periods of elevated activity. These series of attacks were usually triggered by the entry of a non-territorial individual into their territory, or when non-territorial fish, which were up-to that moment mostly inactive, began moving. We only registered aggressive attacks on about 38% of all sampled points, while mostly foraging or resting behavior was observed in the remaining recordings (Fig. 5d).

Hormone analyses

Distinct social statuses within males were also accompanied by different hormonal profiles. Territorial males had on average ten times more 11-KT plasma levels than NT males ($F_{1,6} = 24.03$, $\eta^2_G = 0.70$, $p = 0.005$; Fig. 6a). Circulating levels of testosterone were more than double in T males compared to NT males ($F_{1,5} = 16.43$, $\eta^2_G = 0.22$, $p = 0.010$; Fig. 6b), while the opposite occurred for E_2 ($F_{1,6} = 9.23$, $\eta^2_G = 0.16$, $p = 0.013$; Fig. 6c). Plasma cortisol was found not to vary between T and NT males ($F_{1,6} = 0.12$, $\eta^2_G = 0.01$, $p = 0.742$; Fig. 6d). The ratio $E_2{:}(E_2 + \text{testosterone})$, as an index of testosterone metabolization to E_2, was more than three times higher in NT males than in T males ($F_{1,4} = 12.03$, $\eta^2_G = 0.45$, $p = 0.013$; Fig. 6e), while 11-KT:(11-KT + testosterone) ratio, which indicates the physiological
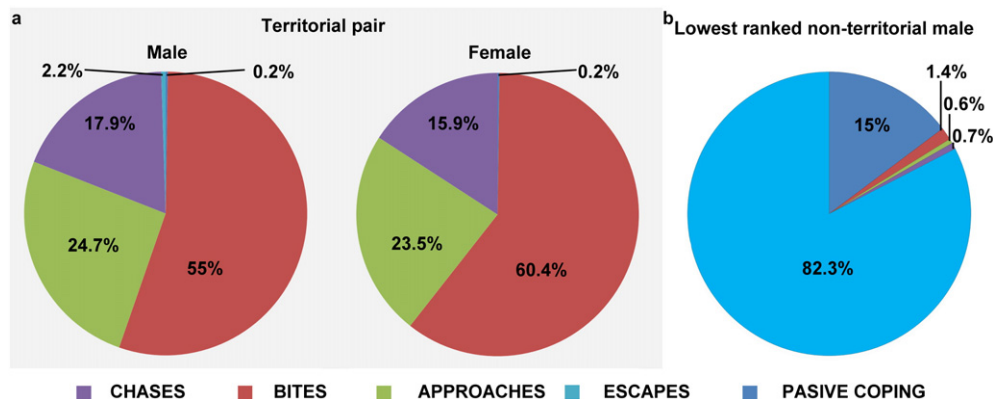
Fig. 3. Social status and behavioral frequencies. Pie charts showing the average frequency of each type of behavior as a percentage of the total number of interactions registered for each fish.

conversion of testosterone to 11-KT, showed a tendency to be higher in T males ($F_{1,5} = 5.84$, $\eta^2_G = 0.37$, $p = 0.072$; Fig. 6f).

The index of agonistic behavior positively correlated with male 11-KT plasma levels (Pearson's correlation coefficient $= 0.79$, $r^2 = 0.63$, $p = 0.0028$; Fig. 7a) and negatively with E_2 concentration (Pearson's

correlation coefficient $= -0.58$, $r^2 = 0.34$, $p = 0.039$; Fig. 7c). No correlation was found between the index of agonistic behavior and testosterone ($p = 0.13$; Fig. 7b) or cortisol ($p = 0.46$; Fig. 7d) plasma levels. The index of testosterone conversion to 11-KT was strongly and positively associated with the index of agonistic behavior (Pearson's correlation coefficient $= 0.72$, $r^2 = 0.51$, $p = 0.014$; Fig. 7e), but such was not the case for its conversion to E_2 ($p = 0.085$; Fig. 7f).

Quantification of testicular cell types

Differences between T and NT males were also present at the testes level. Territorial males had proportionally larger testis than NT males as evidenced by their higher GSI ($F_{1,6} = 17.41$; $\eta^2_G = 0.20$; $p = 0.006$; Fig. 8a). Within T male testes, spermatozoa dominated the cellular landscape with an estimated relative abundance of 34.5%, whereas spermatocytes were the major cellular component within NT males' testes (39.1%). The comparison between males of different social status revealed that the percentages of spermatocytes ($F_{1,6} = 9.32$; $\eta^2_G = 0.42$; $p = 0.031$; Fig. 8b) and spermatids ($F_{1,6} = 10.15$; $\eta^2_G = 0.69$; $p = 0.031$; Fig. 8b) were higher in NT males, while T males showed a greater percentage of spermatozoa ($F_{1,7} = 60.53$; $\eta^2_G = 0.47$; $p = 0.0005$; Fig. 8b).

Discussion

In the present work we further characterized *C. dimerus* social structure with emphasis on pair cooperative territoriality, described the physiological profiles associated with social status and analyzed the existence of reciprocal relationships between individual variation in agonistic behavior and variation in sex steroids and cortisol plasma levels. We found that T fish can be identified early during hierarchy development. We also noticed that males of different social status differed in their hormonal profiles (Fig. 6), and that variation in androgen and estrogen plasma levels positively and negatively correlated with interindividual variation in agonistic behavior (Fig. 7), respectively. Dissimilarities were observed also at the level of testes, as T males were more prepared for reproduction, denoted by a higher GSI and spermatozoa percentage (Fig. 8).

When animals compete for finite resources such as food, territory or mates, social interactions determine which animal gains access to the contended assets (Svare, 1983). When we placed a group of six size-matched *chanchitas* within the experimental tanks, the area around the flat slate became a valuable resource, as it is usually chosen as a spawning site. The size of the employed tanks was just enough for the establishment of a single territorial pair, probably turning competition even stronger (Alonso personal communication). The pattern of pair formation was in accordance with the typical behavior shown by paired territorial cichlids (Baerends and Baerends-Van Roon, 1950) and resembled that observed in other Neotropical cichlids like *Amatitlania nigrofasciata* (Mackereth and Keenleyside, 1993) and *Bujurquina vittata* (Timms and Keenleyside, 1975), and the North American cichlid *Herichthys cyanoguttatus* (Haley, 1987), in which the most dominant fish of each sex within a dominance hierarchy, were the first to form pairs.

Twenty-four hours after the introduction of the fish to the experimental setup, individuals interacted with each other by means of overt aggression and threatening displays. The latter included opening
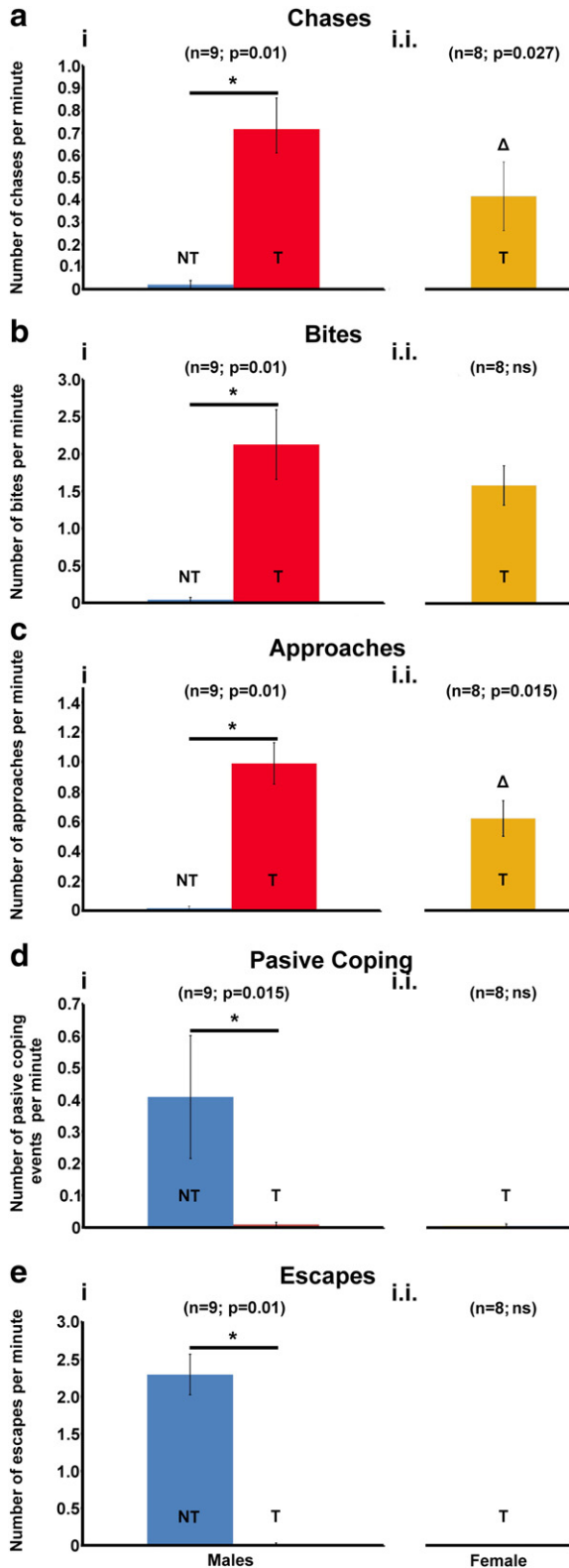

Fig. 4. Social status and behavioral repertoire. Territorial (T) males performed more (a.i.) chases, (b.i.) bites and (c.i.) approaches than the lowest ranked non-territorial (NT) males, while the frequency of submissive behaviors, (d.i.) passive coping and (e.i.) escapes, were higher in NT males. Territorial females performed less (a.i.i) chases and (c.i.i) approaches than territorial males. No differences were observed in the frequency of (b.i.i.) bites, (d.i.i) passive coping and (e.i.i) escapes between T male and female. Asterisks indicate statistical significance between males of different social status, while triangles point out significant differences in territorial male and female behavioral frequencies.
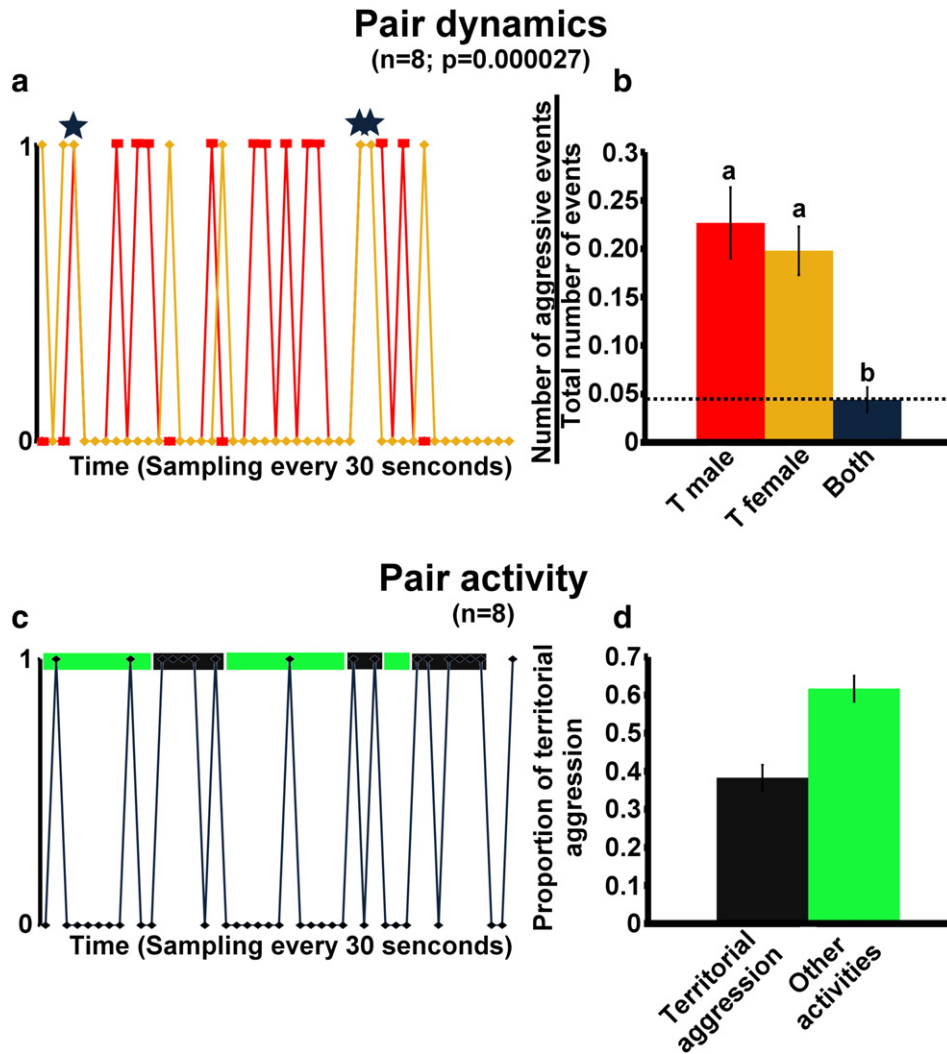
Pair dynamics
(n=8; p=0.000027)

Pair activity
(n=8)

Fig. 5. Pair dynamics. (a) Example of a one-zero sampling where we registered the occurrence of territorial aggression by either the territorial male (red line — squares), territorial female (yellow line — rhombuses) or both (signposted with a star). (b) Proportion of aggressive events over total number of sampling points (one every 30 s) for territorial (T) male and female, or join action of the pair (both). The dashed line at 0.045 shows the expected likelihood of the pair attacking simultaneously by chance (frequency of male territorial aggression × frequency of female territorial aggression). Different letters denote significant differences (p = 0.000027, data were square root-transformed; Randomized Block Design Analysis of Variance). (c) Example of a one-zero sampling where we recorded the occurrence of aggression by the territorial pair (blue rhombuses, male and female together), once every 30 s. (d) In about 38% of all sampled points we observed aggressive attacks by the territorial pair, while mostly foraging or resting behavior dominated most of the recordings.

of the opercula, fin erection, parallel swimming and tail swipes, as also observed in other Neotropical cichlids (Rogers, 1988; Teresa and Goncalves-de-Freitas, 2011; Timms and Keenleyside, 1975). These non-physical threats allow the assessment of rivals without the risk of sustaining high cost injuries. Once the linear hierarchy was established only overt aggression persisted, as evaluation of other community members might have not longer been necessary. Instead, a reinforcement of relative rank seemed to occur through bites, chases, approaches, escapes and passive coping.

As seen in other cichlid species (Fernald and Hirata, 1977; Oliveira and Almada, 1996), as well as previously described in *C. dimerus* (Alonso et al., 2011), the lowest ranked non-territorial *chanchitas* rarely performed aggressive behavior (Figs. 3 and 4). This became unidirectional, directed from dominant to subordinate fish, which limited their agonistic repertoire to escaping from and passively coping with attacks by other *chanchitas* (Figs. 3 and 4). This asymmetrical social structure may result beneficial for both T and NT males, as all fish experience a reduction in the potential costs of continuous fighting (Gurney and Nisbet, 1979). Nonetheless, NT males were also socially denied access to reproduction, thus this short-term energy saving may turn irrelevant to

overall animal fitness (Sloman and Armstrong, 2002). In the long run, however, evidence from other cichlids suggests that this energy saving may result beneficial for NT males, if allocated to growth. In the African cichlid *Astatotilapia burtoni*, NT males grew faster than T males (Hofmann et al., 1999). This reallocation of resources towards somatic growth increases the chance of NT males becoming territory-holders when an opportunity becomes available.

In regard to the dynamics of the territorial pair, we found that males performed more chases and approaches than T females, and that they rarely attacked in synchrony (Figs. 4 and 5). These observations agree with those described for most monogamous Neotropical cichlids with biparental care, which involves male and female cooperatively guarding a territory and brood with division of labor (Itzkowitz et al., 2002; McKaye and Murry, 2008; Snekser and Itzkowitz, 2009). This mode of action allows territorial animals to alternate periods of vigilance with feeding and resting activities, without sustaining elevated threat to territory lost, while at the same time maintaining better health conditions (Perrone and Zaret, 1979). The fact that females performed fewer chases and approaches than males, points out for an unequal division of tasks, as males may be more involved in territory defense, whereas
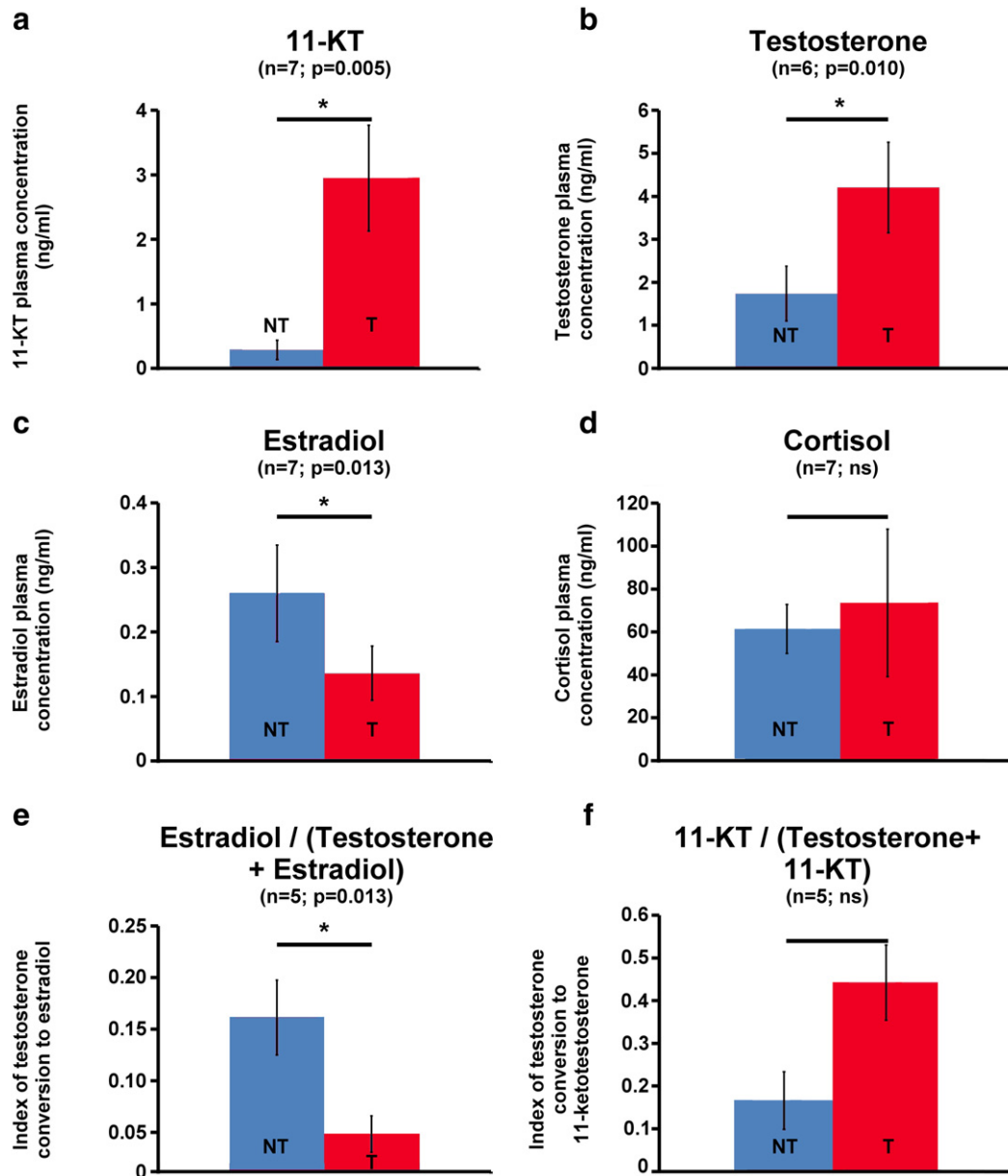
Fig. 6. Social status and hormone profiles. Territorial (T) males had higher (a) 11-ketotestosterone (11-KT, log-transformed data) and (b) testosterone plasma levels than the lowest ranked non-territorial (NT) males. NT males' (c) 17β-estradiol plasma levels and (e) testosterone conversion to 17β-estradiol were higher than in T males. (d) Blood cortisol (log-transformed data) did not differ between statuses, while (f) testosterone conversion to 11-KT showed a tendency to be higher in T males (p = 0.072). The asterisk indicates statistical significance.

females might show a higher investment in preparation of the spawning site.

As with agonistic behavior, sex steroids also differed between males of distinct social status. Territorial males had higher androgen plasma levels (testosterone and 11-KT) compared to the lowest ranked NT males (Fig. 6a and b). This is in agreement with the strong association observed between androgens and dominance in a large number of vertebrates (Parikh et al., 2006; Taves et al., 2009). In fact, there are multiple studies in which androgen removal leads to a decrease in aggressive behavior, which is later rescued by androgen treatment (Francis et al., 1992; Lincoln et al., 1972; Weiss and Moore, 2004). In chanchita, 11-KT positively correlated with the index of agonistic behavior (Fig. 7a), as higher positive values are indicative of elevated aggression. No such correlation was found for testosterone (Fig. 7b), strengthening the idea of 11-KT as the main bioactive androgen in male teleosts, involved in the regulation of secondary sexual characteristics, reproductive and aggressive behavior (Borg, 1994; Hishida and Kawamoto,

1970). The index of conversion of testosterone to 11-KT showed a tendency to be higher in T males (Fig. 6f) and was positively correlated with agonistic behavior (Fig. 7f), suggesting a possible action of the social environment on the metabolic machinery involved in 11-KT biosynthesis. However, in the haremic bidirectionally hermaphroditic fish, Lythrypnus dalli, blocking 11-KT synthesis specifically within the brain did not alter male agonistic performance, but rapidly reduced parental behavior (Pradhan et al., 2014). The latter work along with the findings in this study, suggest that plasma 11-KT involved in the regulation of at least some components of aggressiveness, might be of gonadal origin. Accordingly, castration of A. burtoni led to a decrease in aggression but not of social dominance (Francis et al., 1992), highlighting the importance of the analysis of individual variation in behavior and not just social status.

Historically, there has been a supported notion that male to male aggressive behavior was mainly regulated by androgens (Simon, 2002). However, in the past decades E₂ has emerged as a key element in the
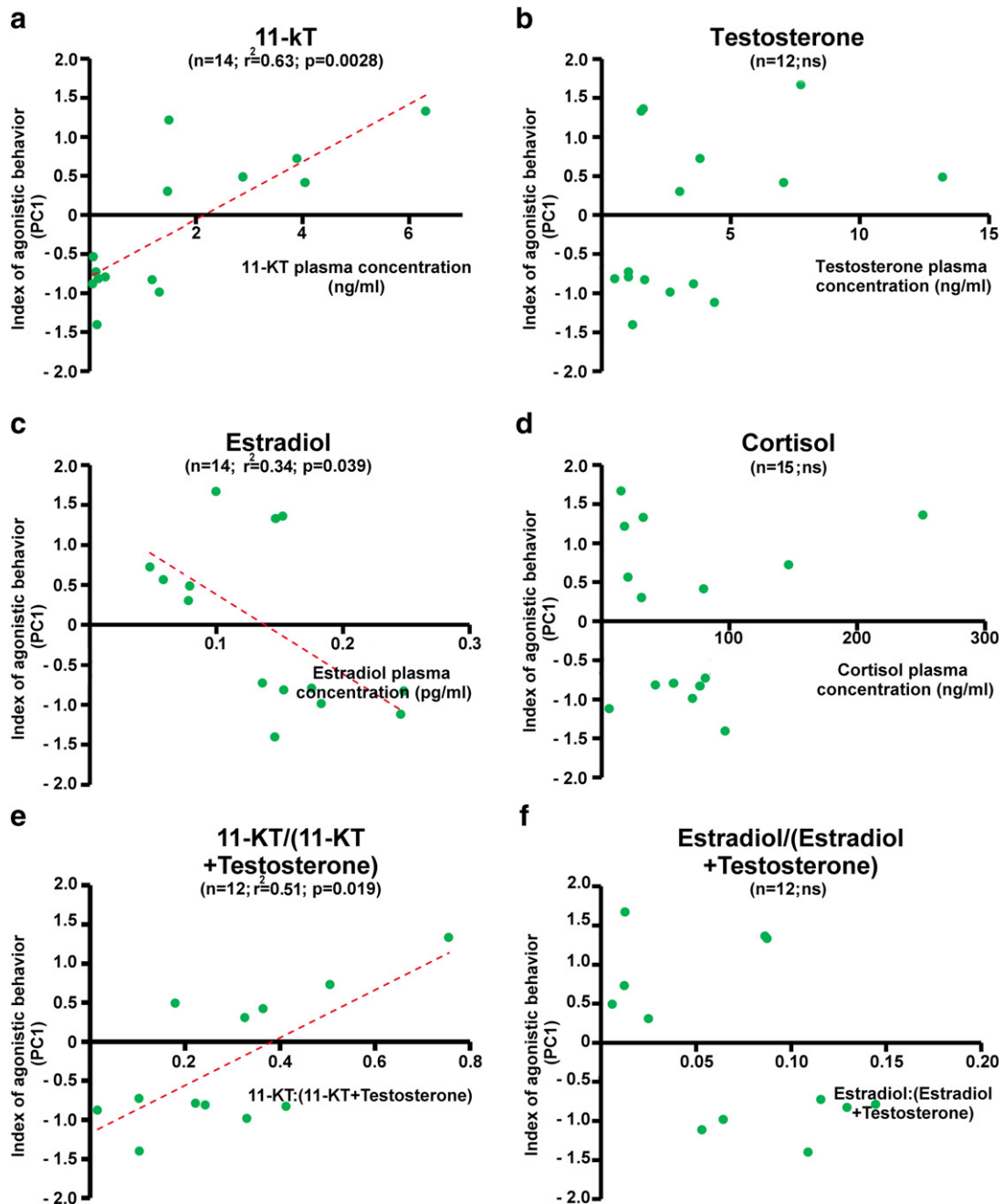
Fig. 7. Hormones and agonistic behavior. (a) 11-KT (11-ketotestosterone) was positively correlated with the index of agonistic behavior (PC1: First principal component), while (c) 17β-estradiol showed the opposite pattern. No correlation was observed for (b) testosterone and (d) cortisol. (e) Testosterone metabolic conversion to 11-KT was positively associated with PC1, while no association was found for the (f) index of testosterone conversion to 17β-estradiol. Higher positive PC1 values are associated with stronger territorial aggression, whereas more negative values indicate an elevated frequency of submissive behavior. Coefficient of determination, r^2.

regulation of male reproductive and aggressive behavior (Huffman et al., 2013; Ogawa et al., 2000; Soma et al., 2000b; Schlinger and Callard, 1990). In *chanchita*, E_2 plasma levels were higher in the non-aggressive NT males (Fig. 6c), and it negatively correlated with the index of agonistic behavior (Fig. 7c); i.e. E_2 showed an association with an elevated frequency of submissive behavior and a low frequency of aggressiveness. Similar associations were observed in many studies where a reduction in aggressive behavior resulted from treatment with E_2 or estrogen analogues (Bell, 2001; Filby et al., 2012; Gonçalves et al., 2007; Marsh-Hunkin et al., 2013). However, the exact opposite has been observed in *A. burtoni*, where T males presented elevated E_2 plasma levels compared to NT males (Maruska and Fernald, 2010a; O'Connell and Hofmann, 2011), and treatment with E_2 increased the

frequency of aggressive displays independent of status (O'Connell and Hofmann, 2011). These differences cannot be explained by the degree of hierarchy stability, as in O'Connell and Hofmann (2011) the employed *A. burtoni* males maintained their social status for 1 week, similar to our study. Also, in males transitioning from NT to T, there is a quick surge in E_2 plasma levels within 30 min of social ascend opportunity (Maruska and Fernald, 2010b). A possible explanation could lay, however, in the distinct reproductive systems of *A. burtoni* and *C. dimerus*. The former presents a lek-like system with female mouth-brooding, while *C. dimerus* is a monogamous species with bi-parental care of the egg and larvae. Some studies have reported a negative effect of paternal behavior over E_2 plasma levels (Knapp et al., 1999; Specker and Kishida, 2000), such that pre-spawning *C. dimerus* E_2 concentration
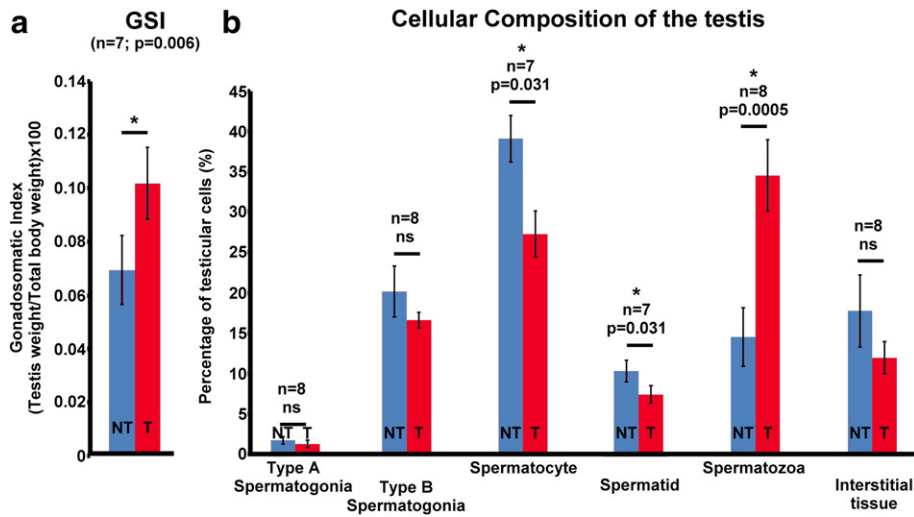
Fig. 8. Social status and testicular morphology. (a) Territorial (T) males gonadosomatic index (GSI) was 31.7% higher than that of the lowest ranked no-territorial (NT) males. (b) At a cellular level, T male testis showed a larger percentage of spermatozoa than that of NT males, while spermatocyte and spermatid percentage was greater in NT males. Data from type B spermatogonia were cos-transformed.

could represent an initial decline in anticipation of the onset of parental behavior.

As in *A. burtoni*, studies in other vertebrates also point out a positive causal link between E_2 and aggression (Huffman et al., 2013; Soma et al., 2000b; Trainor et al., 2008; Zumpe et al., 1993). Yet, half of these studies focused on aggression outside the reproductive season or under a short photoperiod, when the neural mechanisms involved in the regulation of aggressive behavior seem to differ from that of the reproductive season (e.g. androgen independent; Soma et al., 2000a). The other half analyzed the effect on aggression of blocking E_2 synthesis by inhibition of aromatase, the key enzyme involved in the conversion of testosterone to E_2. Aromatase activity and gene expression within the brain are differentially regulated in distinct neural regions linked to social and reproductive behavior, where E_2 exerts a local effect (Dickens et al., 2014; Huffman et al., 2013; Remage-Healey et al., 2008; Ubuka et al., 2014). Thus, treatment with an aromatase inhibitor impairs E_2 synthesis in all brain regions, including local regulatory niches of aggressive behavior. Conversely, no variation in testicular aromatase mRNA levels was observed between stable dominant and subordinate *A. burtoni* males (Maruska and Fernald, 2011). But in the current work, we measured E_2 in the general circulation by caudal vein puncture, which does not necessarily reflect local changes in estrogen synthesis within the brain. Accordingly, we found indirect signs that the overall index of testosterone conversion to E_2 was higher in NT males (Fig. 6e), yet it did not correlate with the index of agonistic behavior (Fig. 7e). These data suggest that (1) circulating plasma E_2 might be the result of various differentially regulated sources, (2) that male status, but not agonistic behavior, is probably associated with overall aromatase activity, (3) that in NT males there is a higher availability of testosterone for its conversion to E_2, as its metabolization to 11-KT is extremely reduced, or (4) that aggressiveness and submissiveness are not two extremes of a single behavior (combined in the index of agonistic behavior; PC1), and thus should be analyzed separately. In this regard, we found that the index representative of the frequency of submissive behavior by itself positively correlated with E_2 plasma levels (Pearson's correlation coefficient = 0.77, r^2 = 0.59, p = 0.002), while the index of aggressive behavior showed a trend for a negative relationship (Pearson's correlation coefficient = −0.52, r^2 = 0.27, p = 0.057). None of the behavioral indexes correlated with the index of testosterone conversion to E_2. This provides evidence for a distinct regulation of submissive and aggressive behaviors, and that submissiveness should not simply be regarded as the absence of aggression. Furthermore, in the cooperatively breeding African cichlid *Neolamprologus pulcher*, E_2 plasma levels of resident

males and females subjected to a stimulated territory intruder protocol remained constant, despite an increase in the frequency of aggressive acts (Desjardins et al., 2006), which supports the hypothesis that E_2 concentration in the general circulation is not a good predictor of aggression.

We found that cortisol did not vary between males of different social status (Fig. 6d). This is in disagreement with the general notion that subordination is associated with higher stress levels, showcased through elevated plasma glucocorticoids. Increased cortisol secretion is a primary indicator of stress in teleost fish (Pankhurst, 2011), as in many other vertebrates species (Baker et al., 2013), including *C. dimerus* (Alonso et al., 2011; Morandini et al., 2014). However, stress can be experienced by both top- and bottom-ranked animals, and is dependent on social organization, composition and individual variability (Creel, 2001; Sapolsky, 2005). For instance, wild savannah babbons (*Papio cynocephalus*) exhibit a hierarchical linear system in which glucocorticoids levels were lower in high ranking males, except for the highest ranked *alfa* male. The latter showed comparably high stress hormone levels to males in the lower part of the hierarchy, probably associated with an elevated cost of continuous fighting and mating (Gesquiere et al., 2011). Similarly to what we observed in *chanchita*, in the highly social *A. burtoni*, size-matched dyads of socially stable dominant and subordinate males did not differ in their plasma cortisol levels (Fox et al., 1997; Maruska and Fernald, 2010a). In the case of *chanchita*, NT male hormonal stress could be associated with the monopolization of breeding by T individuals through overt aggression and intimidation. Whereas T males' similar cortisol levels might result from constantly defending the territory from size-matched rivals and quite possibly, due to the proximity to spawning, as cortisol appears to increase around spermiation (Castranova et al., 2005; Scott et al., 1984). Accordingly, in *N. pulcher*, dominant individuals had higher cortisol levels than subordinates, most likely resulting from the difficulty in obtaining and maintaining a breeding position via competition, but also as a result of gametes production in preparation for spawning (Mileva et al., 2009). Also, in Alonso et al. (2011) hormonal variability associated with social status was studied in groups of *chanchitas* which were kept together for longer periods of times (at least one more week). Thus, in the current work hierarchy stability may be too recent, and T male cortisol levels might be reminiscent of previous hierarchy development. Accordingly, when Fox et al. (1997) studied the cortisol dynamics of *A. burtoni* T and NT males housed in community tanks, they found that as an index of hierarchy stability increased, significant differences between T and NT cortisol levels became apparent.

It is noteworthy that T males showed considerable variation in their cortisol and 11-KT plasma levels. This could be the result of intrinsic variability among fish (Kempenaers et al., 2008; Williams, 2008), differences in the readiness to spawn at the time of fish analysis (Kindler et al., 1989; Liley et al., 1986; Scott et al., 1984), but also due to the heterogeneous social environment each fish experienced (DeVries et al., 2003; Oliveira, 2004). As we could not distinguish between male and female *C. dimerus*, all experimental setups possibly differed in the proportion of sexes. The compositions of the social environment (Lacava et al., 2011; Oliveira et al., 1996), as well as fish previous social status (Oliveira et al., 2009) and age (Alcazar et al., 2014) have all been shown to affect an animal's physiology and the likelihood of becoming T or NT. Thus, in future studies we must reduce social heterogeneity as much as possible, by placing not only size-matched, but individuals with similar backgrounds and experience.

The social scenario did not only shape the endocrine landscape (and/or vice versa), but also pertain an effect on testicular morphology. Territorial *C. dimerus* had larger testis relative to body size than NT males (Fig. 8a) and showed a higher percentage of spermatozoa (Fig. 8b). The same pattern in GSI associated with social status has been found in other cichlids like *Oreochromis niloticus* (Golan and Levavi-Sivan, 2013), *Oreochromis mossambicus* (Oliveira and Almada, 1998), *A. burtoni* (Maruska and Fernald, 2011) and *A. nigrofasciata* (Chee et al., 2013), where T males also presented higher GSI compared to the physiologically suppressed NT males, highlighting a common evolutionary response to social suppression, resulting in a reduced reproductive investment. Accordingly, in NT male *chanchitas*, a higher GSI correlated with a higher dominance index (Alonso et al., 2012). Larger testes are also in agreement with the described steroid hormone profiles, as higher 11-KT levels observed in T males are probably associated with stimulation of Sertoli cells, which in turn promote spermatogonial proliferation, meiosis and spermiogenesis (Miura et al., 1991). On the other hand, the constant intimidation and attacks executed by fish of higher rank over the lowest-ranked NT males, exerted a sort of "social contraceptive" which seemed to delay, rather than completely impair, spermatogenesis. This social constraint was particularly strong at the late stages of spermatogenesis, during final spermatozoa maturation, as spermatocytes and spermatids accumulated within NT males' testis. Yet NT males still possessed cyst of every spermatogenic cell type, which points for a still ongoing spermatozoa development. Thus, even though T males were better suited for immediate reproduction, NT males still hold reproductive potential. This could be of particular importance if social structure becomes unstable, and the opportunity for social ascent emerges. This would be in agreement with the notion suggested by Maruska and Fernald (2011), that males transitioning from subordinate to dominant status undergo a reactivation of an already functional reproductive axis (brain–pituitary–gonads), similar to pubescent mammals. It would be of great interest to further analyze the dynamics in spermiogenesis reactivation and the endocrine and molecular processes involved, as a NT male suddenly becomes territorial.

Conclusions

C. dimerus is a social species in which male and female form territorial pairs that cooperatively, and through an intercalated fashion, defend a territory from potential intruders. Male social position within a stable linear hierarchy impacts on its behavior, steroid levels and testicular development. Territorial males display higher levels of aggression, in correlation with androgen and estrogen levels, and are better suited for reproduction, denoted by an elevated spermatozoa content and larger relative testis size.

Acknowledgments

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