

Photoperiod modulation of aggressive behavior is independent of androgens in a tropical cichlid fish



Eliane Gonçalves-de-Freitas^a, Thaís Billalba Carvalho^b, Rui F. Oliveira^{c,d,*}

^a Universidade Estadual Paulista e Centro de Aquicultura da UNESP, R. Cristóvão Colombo 2265, 15054-000 São José do Rio Preto, SP, Brazil

^b Universidade Federal do Amazonas, Av. Gal. Rodrigo Octávio Jordão Ramos 3000, Manaus, AM, Brazil

^c ISPA – Instituto Universitário, R. Jardim do Tabaco 34, 1149-041 Lisboa, Portugal

^d Champalimaud Neuroscience Programme, Instituto Gulbenkian de Ciência, Rua da Quinta Grande 6, 2780-156 Oeiras, Portugal

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ABSTRACT

Photoperiod is a major environmental cue that signals breeding conditions in animals living in temperate climates. Therefore, the activity of the reproductive (i.e. hypothalamic–pituitary–gonadal, HPG) axis and of the expression of reproductive behaviors, including territoriality, is responsive to changes in day length. However, at low latitudes the seasonal variation in day length decreases dramatically and photoperiod becomes less reliable as a breeding entraining cue in tropical species. In spite of this, some tropical mammals and birds have been found to still respond to small amplitude changes in photoperiod (e.g. 17 min). Here we tested the effect of 2 photoperiod regimes, referred to as long-day (LD: 16L:08D) and short-day (SD: 08L:16D), on the activity of the HPG axis, on aggressive behavior and in the androgen response to social challenges in males of the tropical cichlid fish *Tilapia rendalli*. For each treatment, fish were transferred from a pre-treatment photoperiod of 12L:12D to their treatment photoperiod (either LD or SD) in which they were kept for 20 days on stock tanks. Afterwards, males were isolated for 4 days in glass aquaria in order to establish territories and initial androgen levels (testosterone, T; 11-ketotestosterone, KT) were assessed. On the 4th day, territorial intrusions were promoted such that 1/3 of the isolated males acted as residents and another 1/3 as intruders. Territorial intrusions lasted for 1 h to test the effects of a social challenge under different photoperiod regimes. Photoperiod treatment (either SD or LD) failed to induce significant changes in the HPG activity, as measured by androgen levels and gonadosomatic index. However, SD increased the intensity of aggressive behaviors and shortened the time to settle a dominance hierarchy in an androgen-independent manner. The androgen responsiveness to the simulated territorial intrusion was only present in KT but not for T. The percent change in KT levels in response to the social challenge was different between treatments (SD > LD) and between male types (resident > intruder). The higher androgen response to a social challenge in residents under SD may be explained by the time course of the androgen response that due to the long time it takes to fight resolution under LD, might have been delayed. This result illustrates the importance of incorporating time response data in social endocrinology studies.

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1. Introduction

Photoperiod plays a key role as an environmental cue signaling the breeding season in temperate species, including fish (Borg, 2010; Bromage et al., 2001; Pankhurst and Porter, 2003). Therefore, it has a profound effect on reproductive and aggressive behaviors, which is mediated by the hypothalamic–pituitary–gonadal (HPG)

axis. In general, long days (LD) stimulate the activity of the HPG axis and the expression of reproductive and aggressive behaviors whereas short days (SD) depress them (see (Nelson et al., 2010) for recent reviews in different taxa). Thus, for animals living in high-latitudes aggressive interactions generally increase as the breeding season approaches and this seasonal effect is mediated by photoperiod. However, the amplitude of the photoperiod seasonal cycle decreases towards the tropics and close to the equator only slight variations are observed. Nevertheless, the tropical environment still presents seasonal variability with alternating dry and rainy seasons, but the year-to-year precision of these seasonal fluctuations is less precise (Hau, 2001). Therefore, tropical species

* Corresponding author at: ISPA – Instituto Universitário, R. Jardim do Tabaco 34, 1149-041 Lisboa, Portugal.

E-mail addresses: elianeg@ibilce.unesp.br (E. Gonçalves-de-Freitas), thaissbillalba@ufam.edu.br (T.B. Carvalho), ruiol@ispa.pt (R.F. Oliveira).

are expected to rely on environmental cues other than photoperiod to entrain reproduction, such as temperature, rainfall, food availability or social factors (Hau, 2001; Pankhurst and Porter, 2003), and to have more flexible responses to changes in local environmental conditions than temperate species (Hau, 2001; Wingfield et al., 1992).

In contrast to the wealth of research on the effects of photoperiod on reproduction and behavior of temperate species, research on tropical species is much more scarce. However, responses to slight fluctuations in photoperiod, in the order of minutes and within the range of photoperiod variation between equinoxes and solstices at tropical latitudes, have been documented in different taxa (e.g. mammals: Heideman and Bronson, 1993; Wayne and Rissman, 1991); birds: (Gwinner and Dittami, 1985; Gwinner and Scheuerlein, 1999; Hau et al., 1998). For example, an increase of photoperiod as small as 17 min is enough to induce gonadal growth in equatorial Spotted Antbirds (*Hylophylax n. naevioides*) (Hau et al., 1998). Therefore, photoperiodic responsiveness may be more present among tropical species than initially expected, and photoperiod may still be used by tropical species as a seasonal cue. Alternatively, animals at equatorial latitudes may be using other solar cues, such as subtle periodic patterns of sunrise and sunset, rather than variation in day length to synchronize their circannual clock (e.g. Goymann et al., 2012).

The reproductive axis and HPG-dependent behavior are also known to respond to social factors. Cichlid fish have been widely studied in this respect (e.g. Fernald and Maruska, 2012; Oliveira, 2009). For example, in the African cichlid *Astatotilapia burtoni* GnRH-immunoreactive neurons in the POA respond to social opportunity with increased activity that drives an increase in circulating androgen levels and dominant behavior in subordinate males ascending in social status (Maruska and Fernald, 2011; Maruska et al., 2010). Thus, socially-driven changes in HPG activity and androgen levels in the breeding context lead to territorial males usually displaying higher androgen levels than non-territorials during the breeding season (Hirschenhauser and Oliveira, 2006; Oliveira et al., 2002). This androgen response to social challenges is moderated by the regime of male intra-sexual competition and by a trade-off with parental care as summarized by the “Challenge Hypothesis” proposed by Wingfield et al. (1990); see also (Goymann et al., 2007; Hirschenhauser et al., 2003). According to this hypothesis, environmental factors that signal the approach of the breeding season, such as photoperiod, are expected to act as anticipatory cues for breeding baseline levels of androgens needed for gonadal development. On the other hand social interactions, such as territorial intrusions or fights for dominance status, are expected to further increase androgen levels towards a physiological maximum, whose function is to adjust the expression of behavior to the local social environment (Oliveira, 2009; Wingfield et al., 1990). The dependence of androgen levels above the breeding baseline on relevant social cues would thus limit the cost associated with keeping androgen levels high for long periods of time (Wingfield et al., 2001). The “Challenge Hypothesis” has received ample evidence across vertebrates (Hirschenhauser and Oliveira, 2006) but see (Goymann et al., 2007), but most studies have been conducted in temperate species. Given the well known latitudinal gradients in biodiversity with maximum species richness in the tropics (Willig et al., 2003), and the contrast between temperate and tropical environments (see above) this variability may limit the universality of the “Challenge Hypothesis”. For example, the costs of keeping elevated testosterone levels during the breeding season in tropical species with long breeding seasons may become unsustainable. Similarly, the higher reliance on local conditions rather than on global entraining cues, such as photoperiod, to signal breeding opportunities in tropical environments, may lead to asynchronous breeding, with reduced

regimes of male–male competition and concomitantly low androgen levels. Studies on tropical birds indicate that they do have indeed lower peak plasma testosterone levels than temperate species (Goymann et al., 2004). For example, in the Spotted Antbird, a tropical species with year-round territoriality, testosterone levels are very low with brief peaks induced by social challenges (Hau et al., 2000), and these low androgen levels are compensated by increased sensitivity of target tissues (Canoine et al., 2006). However, when tropical environments approach the conditions of temperate ones, namely in terms of seasonal territoriality, short breeding seasons or low environmental temperatures, as found in high altitude habitats, higher peak androgen levels are also observed among tropical birds (Goymann et al., 2004). In tropical fish a comparative study on 5 cichlid species differing in mating system and parental care type showed a similar pattern of modulation of the androgen response to social challenges by mating system and parental care as the pattern observed previously described for temperate birds (Hirschenhauser et al., 2004). Finally, in amphibians and reptiles testosterone levels are negatively related to breeding-season length, which may be explained by the higher male–male competition regime expected in shorter breeding seasons (Eikenaar et al., 2012). More studies across different taxa on the response of tropical species to social challenges are still needed.

The redbreast tilapia, *Tilapia rendalli* (Boulenger, 1896), is a good species in which to investigate both the photoperiodic and the social androgen responsiveness of a tropical fish species. It is native to Africa occurring in a latitudinal range from approximately -2° to -25° , which corresponds to a maximum photoperiod amplitude of approximately 3 h, from ca. 10.5L:13.5D at the Winter solstice (June) to ca. 13.75L:10.25D at the Summer solstice (December) at its most southern limit. *T. rendalli* is a monogamous cichlid with substrate spawning breeding where male and female form strong pair bonds (Fryer and Iles, 1972; Ribbink et al., 1981). It has a long breeding season (5–7 months) with multiple spawnings throughout the rainy season (De Bont, 1950; Ribbink et al., 1981). Males are highly territorial during breeding and exhibit offspring defense (Baerends and Baerends-van Roon, 1950; Fryer and Iles, 1972), thus being a good model to test the effects of photoperiod both on the reproductive axis and on aggressive behavior.

We investigated the responsiveness of the reproductive axis and aggressive behavior to changes in photoperiod in *T. rendalli* males, as well as their androgen response to social challenges under short- and long-daylight cycles. If this species is responsive to photoperiod then an increase in androgen levels and aggressiveness is predicted under long-days. Moreover, as a monogamous species, a high amplitude androgen response to a social challenge is predicted by the Challenge Hypothesis, which should be more marked under short-days, when the scope for response (i.e. difference between initial level and maximum physiological response) would be higher, assuming that the maximum physiological level is kept constant across seasons (i.e. different photoperiods).

2. Methods

2.1. Animals and housing

T. rendalli males from Centro Nacional de Pesquisa e Conservação de Peixes Continentais (CEPTA, Pirassununga, SP, Brazil) were used. Fish was kept in an outdoor fish pond (185 m³) at UNESP (São José do Rio Preto, SP, Brazil) until the beginning of the experiment. São José do Rio Preto is located at 20°49' S which falls within the natural latitudinal range of the species, with a natural variation in day length between 10 h 40 min at the Winter

solstice and 13 h 35 min at the Summer solstice. Behavioral experiments were carried on at UNESP from September 2007 to January 2008, and video analysis of behavioral trials and hormonal analysis was subsequently conducted at ISPA – Instituto Universitário (Lisbon, Portugal). Fish were transferred to the laboratory and kept in 500-L indoor tanks (ca.1 fish/10 L) for 15 days before experiment, at 27 ± 1 °C and a 12L:12D light cycle. Food (ration for tropical fish with 32% protein) was provided to satiation twice per day.

2.2. Experimental design

Males were transferred from a pre-treatment photoperiod of 12L:12D into one of two experimental treatments referred as long-day (LD = 16L:08D) and short-day (SD = 08L:16D). Therefore, fish in LD perceived an increase in day length whereas fish in the SD treatment perceived a decrease in day length, and all fish were putatively in a breeding condition before the beginning of the experiment. A day length outside the scope of the natural photoperiod variation was chosen (16L:8D vs. 8L:16D) to maximize the difference between treatments, which can better show the effects of day length on aggressive interactions and androgen responses to social challenge. Animals were grouped in 250 L tanks (ca.1 fish/8 L) for 20 days in each photoperiod and at the end of this period

blood samples were collected to assess initial SD and LD androgen levels. Afterwards animals ($N_{LD} = 13$; $N_{SD} = 8$) were isolated for 4 days in glass aquaria ($40 \times 30 \times 40$ cm, ~44 L) at the end of which a blood sample was drawn to provide androgen levels of non-interacting fish in both photoperiods (i.e. the equivalent to the breeding baseline level in the LD treatment). In parallel, other males were also isolated for 4 days, but in the 4th day they were involved in a single territorial intrusion, either as residents or as intruders. Winning and losing status was recorded, and a second blood sample was taken 1 h after introduction of the intruder to test the androgen response to a social challenge under different photoperiods ($N_{LD} = 13$; $N_{SD} = 10$). Thus, the effects of 3 social contexts on androgen levels were compared under each photoperiod treatment: territorial in isolation, resident and intruder (Fig. 1). The standard length (mean \pm S.E.M.) of the males used in this study was 111.7 ± 0.46 mm and 133.3 ± 0.34 mm for the LD and SD treatments, respectively. The body mass (mean \pm S.E.M.) of the males was 46.57 ± 7.17 g for LD and 80.26 ± 8.17 g for SD. Despite the differences in body size between males allocated to the two treatments it should not influence the endocrine results since there were no correlations between either body mass or standard length and the hormones levels measured either for isolated or for interacting males. Moreover all interacting pairs were formed by size-matched males. Sex was identified by inspection of the genital papillae, as used for Nile tilapia (Mendonça et al., 2010).

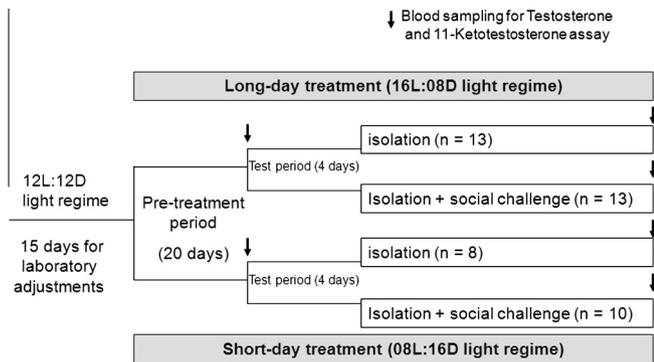


Fig. 1. Experimental design: The *Tilapia rendalli* were kept at 12D:12L regime during 15 days for adjustment to the laboratory conditions in 500-L tanks. Afterwards, fish were assigned to one of two photoperiods: 16L:08D (upper side of the scheme) or 08L:16D (underside) in 250-L tanks to establish a standard pre-treatment period. Then fish were isolated for 4 days in glass aquaria in order to establish territories. On the 4th day, social challenge were promoted by territorial intrusions, such that isolated males acted as residents, as intruders or stayed in social isolation for control. Territorial intrusions lasted for 1 h to test the effects of a social challenge under different photoperiod regimes. Arrows indicate time of blood sampling to assay initial and social levels of androgens.

2.3. Aggressive behavior

Agonistic behavior was video-recorded and subsequently quantified according to the ethogram presented in Table 1. The frequency of attacks was computed as the sum of the overt aggression behavioral patterns, and the frequency of displays as the sum of threatening displays (see Table 1). The intensity of attacks and displays was computed as the number of occurrences of each of them divided by the fight duration time (in seconds). The territorial intrusion was considered to be solved when one of the fish (loser) stopped attacking and started fleeing from the opponent (winner). An escalation index was computed ($EI = \text{number of attacks} / \text{total aggression showed by the pair}$) to compare possible differences on fight escalation between treatments, and a dominance index ($DI = \text{number of victories} / \text{number of victories} + \text{number of defeats}$) was calculated to evaluate the relative asymmetry of each fight (Gómez-Laplaza and Morgan, 1993; Oliveira and Almada, 1996). All behavioral variables were quantified using the multi-event recorder Observer XT (Noldus Inc., Wageningen, The Netherlands).

Table 1
Ethogram of agonistic behavior in *Tilapia rendalli*.

Type	Agonistic act	Description
Attacks	Biting	The aggressor swims towards the opponent and bites its body
	Lateral fight	The fish remain alongside each other facing the same or opposite direction and beat their tails sideways
	Mouth fight	Both fish approach frontally with their mouths opened and bite the opponent's mouth. Their mouths are kept closed while one fish displace the opponent's jaw backward
	Chase	One fish pursues the opponent that swims away
Displays	Tail beating	Only one fish beats its tail sideways
	Lateral threat	The fish approaches the opponent with opened mouth, preparing for lateral biting, but its opponent swims away
	Circling	Two fish with erected dorsal fin swim following each other, describing a circle, like a very slow chasing
Subordination behavior	Flight	The attacked or chased fish swims away from the contest place
	Total aggression	The sum of all agonistic acts but flight

Table 2
Hormone levels (ng/ml plasma) in the pre-test (i.e. before) and test (i.e. after social isolation or social challenge) phases for isolated, resident and intruder *Tilapia rendalli* males. Data are expressed as mean \pm S.E.M.

	Long-day			Short-day		
	Isolated	Resident	Intruder	Isolated	Resident	Intruder
KT before	1.43 \pm 0.18	1.06 \pm 0.11	1.16 \pm 0.21	1.45 \pm 0.36	0.73 \pm 0.19	2.62 \pm 0.85
KT after	12.29 \pm 2.56	7.80 \pm 1.25	4.24 \pm 0.43	16.82 \pm 1.31	12.89 \pm 2.62	5.13 \pm 1.19
T before	1.39 \pm 0.11	1.61 \pm 0.21	1.62 \pm 0.22	1.36 \pm 0.11	1.26 \pm 0.12	2.34 \pm 0.45
T after	6.33 \pm 0.96	4.42 \pm 0.49	3.32 \pm 0.21	8.45 \pm 1.27	5.80 \pm 1.18	2.97 \pm 0.41

2.4. Animal sampling

Biometry, sexing and isolation were preceded by anesthesia with benzocaine (3 mg/L) and blood samples were preceded by immersion in 9 mg/L benzocaine. Fish was considered anaesthetized when detecting loss of gross body movements but regular opercula ventilation (similar to stage II of anesthesia described by Iwama et al. (1989)). The time it took for anesthesia and blood collection was measured for each blood sampling to determine a possible influence of these manipulations on androgen levels. There were no correlations between androgen values and either the time manipulating fish or the time to anesthesia (Pearson correlations, $r < 0.39$, $p > 0.05$). Thus, the hormone analyses for the other sampling points were run. Blood samples were drawn from the caudal vein using a heparin-rinsed needle and syringe. Blood was immediately centrifuged at 2415 g for 10 min and the plasma was stored at -80°C for later analysis. After the last blood sample, fish were sacrificed with an overdose of benzocaine (27 mg/L) and dissected for gonad inspection and confirmation of sexing. Macroscopic analysis of the gonads showed males with broad testes, distended and cream in color, indicative of sexual maturity in *T. rendalli* (Weyl and Hecht, 1998). Gonads were weighed and the gonadosomatic index (GSI) was computed as: $\text{GSI} = (\text{gonad weight}/\text{bodyweight}) \times 100$.

2.5. Hormone assays

Testosterone (T) and 11-ketotestosterone (KT), which is major androgen in teleosts (Borg, 1994), were measured by radioimmunoassay following established protocols (Kime and Manning, 1982; Scott et al., 1984). Intra- and inter-assay coefficients of variation were 1.43 and 2.19 for KT, and 2.17 and 2.36 for T, respectively. The minimum detectable concentrations for the steroids in this study were 0.298 ng/mL for KT and 0.331 ng/mL for T.

2.6. Statistical analysis

Data were checked for outlier values (i.e. mean \pm 2 SD). Three replicates from the LD treatment were outliers for aggressive behavior, but not for hormone values. As a loser (always the intruder) and a winner (always the resident) could be identified in those pairs, we removed the outliers values from aggressive behavior and replaced them by the mean value of the treatment (as recommended by Cousineau and Chartier (2010)), so that sample size remained the same ($N = 13$).

The effect of treatments on the frequency of agonistic behavior, as well as on the escalation index and dominance index was tested using a Two-way ANOVA, with day length (SD vs. LD) and social status (resident vs. intruder) as independent factors. Latency to start the agonistic interaction and the time for hierarchy resolution in LD vs. SD treatments were compared using Student's t -test for independent samples. GSI and initial hormone levels were also compared between treatments (SD vs. LD) by independent Student's t test. The percent change (i.e. the percent variation from

initial to social interaction) of both T and KT levels was compared across treatments using a two-way ANOVA, with social status (isolated vs. resident vs. intruder) and day length (SD vs. LD) as independent factors. The ANOVA assumptions were tested using the Shapiro Wilk's test to check for normality and Levene's test to test homogeneity of variances (Lehner, 1996; Zar, 1999). Androgen percent change and most behavioral variables showed non-normal distribution and thus were square root transformed. A priori planned comparisons were also used to contrast the treatments of interest. Pearson correlations were used to test the association between variation in androgen levels of resident males and behavioral variables. Despite using percent change as a measure of androgen responsiveness we also consider absolute androgen levels to be informative and these are provided (Table 2).

All tests were two-tailed and a $p \leq 0.05$ was used for statistical significance. All statistical analyses were based on Zar (Zar, 1999) and were done on the Statistical Software Package Statistica v.10 (StatSoft Inc.).

2.7. Ethics

This study was conducted according to the ethical guidelines of the Brazilian National Council for the Control of Animal Experiments (CONCEA) and was approved by the Ethical Committee of Animal Experimentation of the São Paulo State University (UNESP, SP, Brazil, permit # 052/06).

3. Results

3.1. Photoperiod effects on the reproductive axis

A 4 h variation in photoperiod (i.e. either from 12L:12D to 16L:8D in LD treatment or from 12L:12D to 8L:16D in SD treatment) over 20 days did not induce a response of the reproductive axis, as measured by either gonadal growth (GSI; LD vs. SD t -test: $t = 1.76$, $df = 65$, $p = 0.09$) or androgen levels (LD vs. SD t -tests: KT, $t = -0.57$, $df = 65$, $p = 0.59$; T, $t = -0.56$, $df = 65$, $p = 0.58$; Table 3).

3.2. Photoperiod effects on aggressive behavior

Latency to start fighting was similar between day lengths (t -test, $t = -0.50$, $df = 21$, $p = 0.62$; Fig. 2A), but agonistic interactions took longer to resolve in LD ($t = 5.85$; $df = 21$; $p < 0.0001$; Fig. 2B). Although the frequency (i.e. number of occurrences/total observa-

Table 3
Reproductive indicators (gonadosomatic index, plasma 11-ketotestosterone and testosterone) of males in pre-test long-day and short-day. Treatments (long-day vs. short-day) were compared using unpaired t tests. Data are expressed as mean \pm S.E.M.

	Long-day	Short-day	t Value ($df = 65$)	p Value
GSI (%)	0.31 \pm 0.04	0.22 \pm 0.02	1.76	0.09
KT (ng/ml)	1.22 \pm 0.10	1.61 \pm 0.35	-0.57	0.59
T (ng/ml)	1.54 \pm 0.11	1.67 \pm 0.19	-0.56	0.58

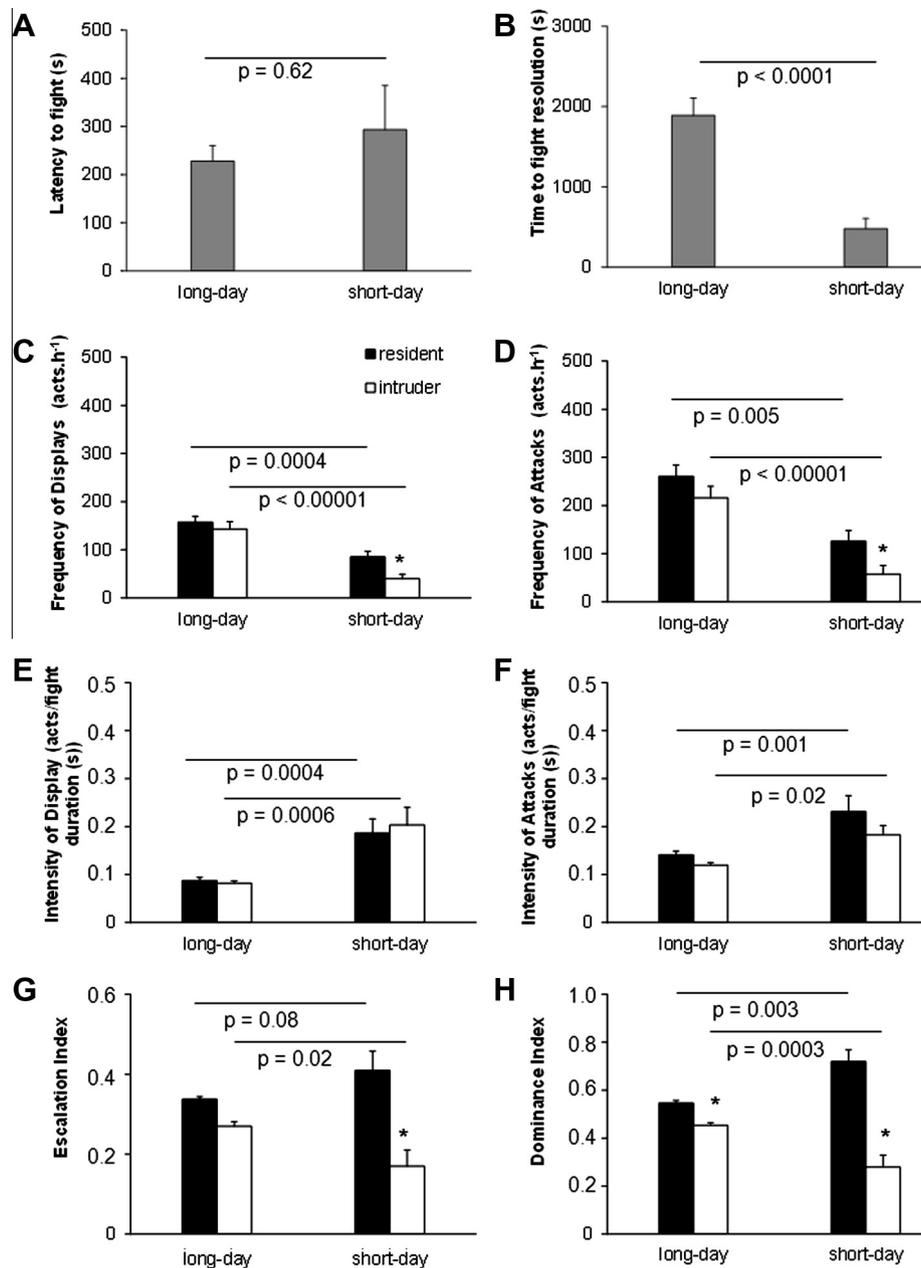


Fig. 2. Behavioral variables measured during the simulated territorial intrusions in long- (LD: 16L:08D) and short-day (08L:16D) treatments: (A) latency to fight; (B) latency to fight resolution; (C) frequency of agonistic displays; (D) frequency of attacks; (E) intensity of displays; (F) intensity of attacks; (G) escalation index; (H) dominance index. Data are expressed as mean \pm S.E.M.; p values on upper line in A and B compare treatments after unpaired t test. p Values on upper lines (C–H) compare residents or intruder between LD vs. SD after planned comparisons. Asterisks indicate significant differences between residents and intruders within each treatment after planned comparisons.

tion time) of both agonistic displays and attacks was higher under LD (Fig. 2C and D) the intensity (i.e. number of occurrences/fight duration) of agonistic displays as well as of attacks was higher in the SD treatment due to the short duration of fights in SD (Fig. 2E and F). There were effects of treatment and rank for the frequency of displays (treatment $F_{1,42} = 55.58$, $p < 0.0001$; rank $F_{1,42} = 9.02$, $p = 0.004$) as well the frequency of attacks (treatment $F_{1,42} = 43.08$, $p < 0.0001$; rank $F_{1,42} = 88.15$, $p < 0.01$). Planned comparisons showed no differences on the frequency of either displays or attacks between resident and intruder fish kept under LD ($F_{1,42} = 0.62$, $p = 0.43$ and $F_{1,42} = 1.48$, $p = 0.23$, respectively). However, residents showed significant higher frequency of displays ($F_{1,42} = 10.90$, $p = 0.002$) and attacks ($F_{1,42} = 7.41$, $p = 0.009$) than intruder fish under SD. There were no interaction effects of social

status and photoperiod treatment for any of these variables (number of displays: $F_{1,42} = 3.85$, $p = 0.06$; number of attacks: $F_{1,42} = 1.55$, $p = 0.22$).

When testing the intensity of agonistic behavior, we found significant effect of treatment for both displays ($F_{1,42} = 23.06$, $p < 0.001$) and attacks ($F_{1,42} = 17.85$, $p < 0.0001$), but there were no effects of the social rank (resident vs. intruder) for those variables (displays: $F_{1,42} = 0.06$, $p = 0.81$; attacks: $F_{1,42} = 3.70$, $p = 0.06$) (Fig. 2E and F). There were no interaction between rank and treatments either for displays ($F_{1,42} = 0.23$, $p = 0.64$) or attacks ($F_{1,42} = 0.55$, $p = 0.46$) intensities.

There was a significant interaction between day length and social rank for both the escalation index ($F_{1,42} = 9.31$, $p = 0.004$) and the dominance index ($F_{1,42} = 30.61$, $p = 0.000002$). Residents

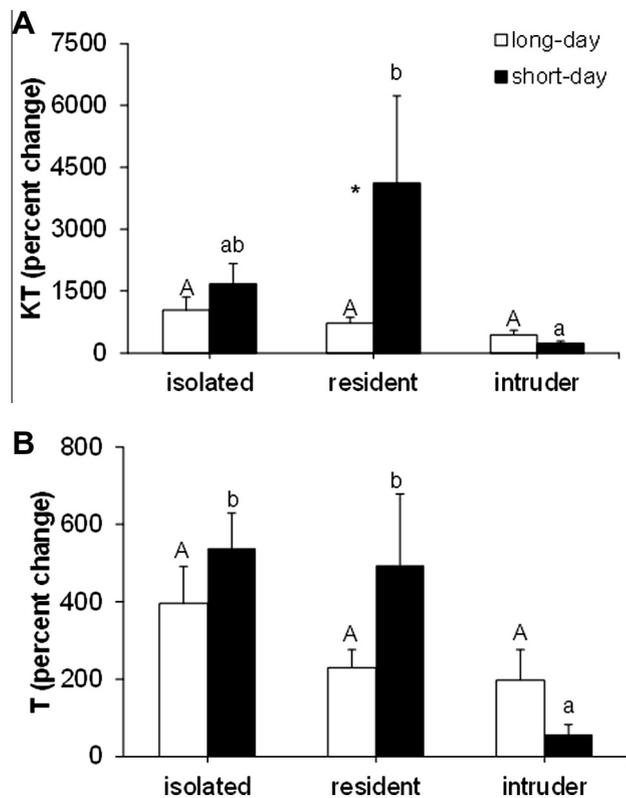


Fig. 3. Percent change of plasma levels of 11-ketotestosterone (A) and testosterone (B) in males in different social context (socially isolated, residents, intruders). Data are expressed as mean \pm S.E.M. Asterisks indicate significant differences between treatments (LD vs. SD) after planned Comparisons. Capital letters compare social contexts (i.e. social isolation vs. resident vs. intruder) within LD and lower case within SD treatment. Significant differences after planned comparisons are indicated by different letters.

and intruders showed similar escalation index ($F_{1,42} = 3.19$, $p = 0.08$) and residents showed higher dominance index than intruder ($F_{1,42} = 5.08$, $p = 0.03$) in LD, but in SD residents showed higher escalation ($F_{1,42} = 31.65$, $p < 0.000001$) and dominance indexes ($F_{1,42} = 87.14$, $p < 0.00001$) than intruders (Fig. 2G and H).

3.3. Photoperiod effects on androgen responsiveness to social challenge

There was a marginally significant main effect of photoperiod treatment (SD > LD) and a significant main effect of social status (resident > intruder) on the KT response to the social challenge ($F_{1,61} = 3.66$, $p = 0.06$ and $F_{2,61} = 3.44$, $p = 0.038$, respectively; Fig. 3A). The interaction between photoperiod treatment and social status was marginally non-significant ($F_{2,61} = 2.78$, $p = 0.069$). In the SD treatment, residents exhibited a higher KT response than intruders ($F_{1,61} = 10.58$, $p = 0.002$, Fig. 3A). However, the KT variation in socially isolated males that were not challenged was not

significantly different from the KT response of intruders ($F_{1,61} = 1.30$, $p = 0.26$, Fig. 3A) and was marginally non-significant when compared to the KT response of residents ($F_{1,61} = 3.70$, $p = 0.059$, Fig. 3A). Within each social status, the difference in KT percent change between SD and LD treatments was significant for residents (SD > LD; $F_{1,61} = 9.09$, $p = 0.004$) and non-significant either for intruder ($F_{1,61} = 0.03$, $p = 0.85$) or isolated males ($F_{1,61} = 0.28$, $p = 0.60$; Fig. 3A).

Neither photoperiod treatment (SD/LD) nor the interaction between photoperiod treatment and social status had significant effects on the T response to the social challenge ($F_{1,61} = 1.25$, $p = 0.27$ and $F_{2,61} = 2.34$, $p = 0.10$, respectively; Fig. 3B). However, there was a significant main effect of social status (resident > intruder) on the percent T change ($F_{2,61} = 6.30$, $p < 0.01$, Fig. 3B). None of the planned comparisons across male types in LD were significant. T response were similar between isolated males and both resident ($F_{1,61} = 0.73$, $p = 0.19$) and intruder males ($F_{1,61} = 2.47$, $p = 0.12$), as well as resident and intruder ones ($F_{1,61} = 0.07$, $p = 0.80$, Fig. 3B). In SD, the percent T change between resident and social isolated fish was non-significant ($F_{1,61} = 0.09$, $p = 0.77$), but both isolated ($F_{1,61} = 9.95$, $p = 0.002$) and resident ($F_{1,61} = 9.21$, $p = 0.04$) showed higher T change than intruders (Fig. 3B). Within each social status, the difference in T percent change between SD and LD treatments was non-significant for isolated ($F_{1,61} = 0.98$, $p = 0.33$) and intruders ($F_{1,61} = 1.07$, $p = 0.031$) and marginally non-significant between residents (SD > LD; $F_{1,61} = 3.82$, $p = 0.055$, Fig. 3B). There were no significant correlations between any of behavioral measures of agonistic behavior and the androgen response to the social challenge for either resident or intruder males (see Table 4 for R values).

4. Discussion

In this study the photoperiod treatments had no effect on the reproductive axis of *T. rendalli*, as measured by GSI and androgen levels. Given this negative result it is important to rule out possible methodological flaws. In fish the timing of reproduction by photoperiod does not depend on a direct relationship to day length, but rather on the degree of compression or extension of the seasonal light cycle in relation to the breeding day length (Borg, 2010; Bromage et al., 2001). Therefore, any day length can be perceived by the fish as long or short, depending on whether the previous photoperiod had been shorter or longer, respectively. This facilitates the occurrence of methodological problems that complicate the interpretation of results when fish are introduced to different experimental photoperiod regimes at different times of the year, or coming from different ambient light regimes (Bromage et al., 2001). In this study, we used a pre-treatment phase with a photoperiod of 12L:12D which lasted for 15 days, before moving the fish to the experimental photoperiod treatments of 16L:8D for LD or 8L:16D for SD. Thus, in our LD treatment fish experienced an increase in day length from pre-treatment conditions, and in the SD a decrease in day length from pre-treatment conditions.

Table 4
Matrix of Pearson's correlations between resident and intruder percent change on androgen levels, and behavioral variables for both long-day and short-day treatments. All correlations were non-significant.

	Long-day (N = 13)					Short-day (N = 10)				
	Attack/s	Display/s	Latency to fight resolution	Escalation index	Dominance index	Attack/s	Display/s	Latency to fight resolution	Escalation index	Dominance index
Resident KT	0.19	0.43	-0.15	0.18	0.50	0.15	-0.28	-0.08	0.09	0.08
Resident T	-0.002	0.26	0.16	0.12	0.39	-0.09	-0.44	0.19	0.38	-0.03
Intruder KT	0.02	-0.04	-0.10	0.03	0.04	0.33	-0.21	0.15	0.13	-0.008
Intruder T	0.10	-0.17	-0.01	0.16	0.11	0.16	-0.13	-0.18	-0.16	-0.08

Moreover, we used day lengths outside the scope of the natural photoperiod of *T. rendalli* (13L:11D vs. 11L:13D for natural LD and SD, respectively) in order to maximize the perceived compression/extension of day length. In summary, the lack of HPG axis response to photoperiod observed in *T. rendalli* does not seem to result from any methodological confound, and must reflect a reliance on other environmental cues to regulate reproductive activity. This result is in accordance with the hierarchical environmental cues hypothesis proposed by Pankhurst and Porter (2003). According to this hypothesis, multiple environmental variables provide cues for fish to enter a reproductive state, and their hierarchy in driving the process varies with latitude. At high latitudes photoperiod and temperature are the primary drivers with local conditions, such as social factors, having a secondary role, whereas in the tropics this hierarchy seems to be reversed (Pankhurst and Porter, 2003).

The lack of effect of photoperiod on the HPG axis in *T. rendalli* presented here contrasts with the enhancing effects of LD on reproductive physiology reported for another cichlid, the South American cichlid *Cichlasoma dimerus* (Fiszbein et al., 2010). A possible explanation for this difference in responsiveness to photoperiod could rely on differences in latitudinal distribution between the two species, since in some other vertebrates the HPG axis of populations living near the equator do not respond to photoperiod (e.g. mammals: (Nunes et al., 2002; O'Brien, 1993). Indeed, despite both species occurring over a large latitudinal range (*T. rendalli* from -2° to -25° in Africa; and *C. dimerus* from -17° to -34° in South America; source for geographical distribution: www.fishbase.com; latitudes were calculated using itouchmap.com), the center of latitudinal distribution of *T. rendalli* is far more equatorial (-13.5°) than that of *C. dimerus* (-25.5°), which is already subtropical.

It should be also noted that in this study fish in both experimental photoperiods (LD and SD) exhibited mature testis. Since the pre-treatment photoperiod of 12L:12D is within the natural photoperiod of this species (LD = 13L:11D; SD = 11L:13D), it is expected that fish entered the experimental photoperiods (LD = 16L:8D; SD = 8L:16D) already in breeding condition. Therefore, the current results should be interpreted as SD being insufficient to arrest reproduction and extended LD being insufficient to further increase the activity of the HPG above a breeding baseline. In another tilapia species, the Nile Tilapia (*Oreochromis niloticus*), only unnatural photoperiods of 6L:6D are effective in arresting reproduction, and these are used in aquaculture settings to control reproduction (Biswas et al., 2005). In this species, extended LD have no effect on female GSI (Rad et al., 2006), but females increase their fecundity (with reduced egg sizes) and reduce the inter-spawn-interval (Campos-Mendoza et al., 2004). Thus, data available in another tilapia species support our results that SD, within a 24 h light cycle, are insufficient to arrest reproduction and that once the reproductive threshold is achieved, photoperiod no longer drives further gonadal allocation.

Contrary to our predictions, despite the lack of HPG axis response to photoperiod, day length affected aggressiveness. Under SD males increased overall aggressiveness in comparison to LD, as measured by the frequency of aggressive displays and attacks in both residents and intruders, indicative of shorter but more intense fights. Interestingly, the longer duration of fights under LD was due to escalation by intruders, suggesting that intruders are more persistent under LD. Finally, the fact that day length did not affect the latency to start the interaction, but fights last longer in LD and are more intense under SD, suggests a lack of effect of photoperiod on basal aggressive motivation, an effect on aggressive persistence by subordinates under LD that extends fight duration, and an effect on aggressive intensity once the interaction is started under SD. The effect of photoperiod on behavioral persistence of intruders under

LD is indicated by the fact that both the escalation index (that measures how much overt aggression was used) and the dominance index (that measures how dominant an animal is over another one) of intruders were higher in LD fights than in SD fights. Indeed, in LD resident and intruder's dominance indexes were significantly different, which was due to the longer time it took for social rank to be established and to the more escalated fights observed in this day length regime.

On the other hand, the increased aggressiveness in SD contrasts with the positive effects of LD on aggressiveness already reported for other teleost species. In the African catfish *Clarias gariepinus*, longer periods of light have been shown to increase aggressiveness in aquaculture settings, with continuous light (i.e. 24L:0D) inducing the highest levels of aggression and complete darkness (i.e. 0L:24D) reducing it substantially (Almazán-Rueda et al., 2004, 2005; Britz and Pienaar, 2009). In the South American cichlid *C. dimerus*, male aggression related to selection of territory is reduced in males exposed to short-days (Fiszbein et al., 2010). In contrast, in the current study the SD induced increase in aggressiveness is independent of an action of photoperiod on the HPG. In fact the present results resemble those obtained in several rodent species, for which a consistent positive effect of SD on male aggression in a resident-intruder paradigm (e.g. Syrian hamsters, *Mesocricetus auratus*: (Caldwell and Albers, 2004; Garrett and Campbell, 1980; Jasnow et al., 2002); Siberian hamsters, *Phodopus sungorus*: (Demas et al., 2004; Jasnow et al., 2000; Wen et al., 2004); beach mice, *Peromyscus polionotus*: (Trainor et al., 2007a); deer mice *Peromyscus maniculatus*: (Trainor et al., 2007b)), is either independent of the HPG axis or paralleled by a regression of the gonads and a decrease in testosterone levels (Caldwell and Albers, 2004; Jasnow et al., 2002; Trainor et al., 2006).

The magnitude of the androgen response to the social challenge was much higher for KT (percent change from initial values up to 4000%) than that of testosterone (below 1000%). This result is in line with data available for other cichlid fish that exhibit a KT, but not a testosterone, response to social challenges (Hirschenhauser et al., 2004). Despite the very high percent change mentioned above the average values of KT and T measured in this study fit the distribution of circulating androgen levels reported for other cichlid species (e.g. (Oliveira et al., 2002; Parikh et al., 2006; Ros et al., 2003). The high amplitude of the KT response to the social challenge observed in resident males is in accordance with the prediction of Challenge Hypothesis that monogamous species have a higher scope of response than polygamous species (Wingfield et al., 1990). This prediction had already been tested in a comparative study on cichlid fish where pair-wise comparisons of closely related species varying in mating system revealed that androgen responsiveness was higher in males from monogamous species, or with only temporal intense pair bonding (Hirschenhauser et al., 2004).

The effect of photoperiod treatment on the androgen response to a social challenge was only significant for KT, for which resident males presented a higher percent change than intruders under SD. This increase in KT in resident males is almost significant when compared to the temporal variation of KT in non-interacting males. Under LD, the KT response of residents to a social challenge is significantly lower than in SD, and it is not different either from the intruders response or from the temporal variation of non-interacting males. Given the absence of photoperiod effect on initial androgen levels and the fact that fights under SD and LD differed in aggressiveness and time to resolution, the most parsimonious explanation for the effect of day length on social androgen responsiveness is that it is a consequence of differential behavioral feedback between photoperiod treatments, rather than a direct effect of photoperiod on androgen responsiveness. Indeed, the behavioral feedback experienced by males in the two treatments differed in

two aspects. A first aspect is that in SD males both expressed and were exposed to higher frequencies of aggressive behavior. According to the Challenge Hypothesis, one would expect the scope of the androgen response to be related to the level of challenge experienced, and therefore given the higher levels of aggression experienced under SD one would thus predict SD also to induce a higher magnitude androgen response (e.g. KT). However, this interpretation is not supported by the lack of correlations between the magnitude of the androgen response and the frequency of aggressive behaviors. A second aspect has to do with the time course of the androgen response. If the triggering of the KT response requires the individual to assess the outcome of the fight, as suggested for other cichlids (Oliveira and Canario, 2011; Oliveira et al., 2005), then given the longer time it took for fights to be solved in the LD treatment, it was only close to the end of the trial that the response was triggered and the blood sampling at the end of the interaction may have missed the peak of the response, which was captured in the SD treatment due to an earlier resolution of the fight. This possibility is the most plausible explanation for our results and stresses the need to incorporate time course of androgen responses in future studies.

Finally, the fact that isolated fish, used as a control treatment for the social interactions, also presented an increase in androgen levels during the experimental period can be seen as surprising, since this response cannot be due to agonistic interactions. This result may lead to the conclusion that the observed increase in androgen levels in residents under SD similarly cannot be attributed to the conflict, as suggested above. However, previous studies on the effects of social isolation on androgen levels in cichlid fish have shown that social isolation can be perceived as a gain in social status (i.e. becoming territorial) in fish that lack a territory before social isolation (Galhardo and Oliveira, 2014). Since in the pre-treatment phase fish were kept at a density that prevented them to establish territories, we interpret the rise in androgen levels induced by social isolation as a result of fish perceiving an opportunity to become territorial. Therefore, the observed increase in androgens in isolated fish and in residents of the SD treatment most probably had different proximate causes.

In summary, day length had no effect on GSI or initial androgen levels and thus does not seem to be used by *T. rendalli* as an environmental cue for HPG axis regulation. However, day length had an effect on aggressiveness with SD eliciting more aggressive responses to simulated territorial intrusions. Finally, socially driven androgen responses are present in this species, confirming the Challenge Hypothesis for another tropical fish species.

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