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The effects of social isolation on steroid hormone levels are modulated by previous social status and context in a cichlid fish

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ABSTRACT

Social isolation is a major stressor which impacts the physiology, behaviour and health of individuals in gregarious species. However, depending on conditional and contextual factors, such as social status and group composition, social isolation may be perceived differently by different individuals or even by the same individuals at different times. Here we tested the effects of social status (territorial vs. non-territorial) and previous group composition (i.e. type of social group: mixed sex group with two territorial males, TT vs. mixed sex group with one territorial and one non-territorial male, TnT) on the hormonal response (androgens and cortisol) to social isolation in a cichlid fish (*Oreochromis mossambicus*). The different steroid hormones measured responded differentially to social isolation, and their response was modulated by social factors. Social isolation elicited a decrease of 11-keto formation only in territorial males, whereas non-territorial males present a non-significant trend for increasing KT levels. Testosterone did not respond to social isolation. Cortisol only increased in isolated individuals from TnT groups irrespective of social status (i.e. both in territorials and non-territorials). These results suggest that it is the perception of social isolation and not the objective structure of the situation that triggers the hormonal response to isolation.

Introduction

The social environment plays a key role in the life of group-living species, having a major impact on many biological processes including brain and behaviour (Fernald, 2012; Fernald and Maruska, 2012). Individuals from social species are motivated to establish and maintain social connections, showing a preference for being in the presence of conspecifics and valuing them as social rewards (e.g. fish: Al-Imari and Gerlai, 2008; mammals: Thiel et al., 2008). On the other hand, in social species deprivation from social conspecifics (i.e. social isolation) is a major stressor, which impacts the physiology, behaviour and health of individuals (Cacioppo et al., 2011; Hennessy, 1997). Detrimental effects of social isolation seem to be universal across different animal taxa, as they have been documented from fruit flies, in which social isolation decreases lifespan (Ruan and Wu, 2008), to mammals (e.g. mice, rat, rabbit, and humans), in which it is associated with increased hypothalamic–pituitary–adrenal (HPA) axis activation, increased sympathetic activity, increased oxidative stress, immunosuppression, lack of inflammatory control, and sleep disturbances (for a brief review see Cacioppo et al., 2011). Interestingly, in humans these detrimental effects are not only associated with real social isolation but are also triggered simply by the perception of social isolation (i.e. loneliness), and together contribute to higher rates of morbidity and mortality in older adults (Cacioppo et al., 2011). In fish, social isolation has been shown to impact locomotion, exploratory behaviour, feeding, aggressive behaviour and androgen and corticosteroid levels (Gómez-Laplaza and Morgan, 2000, 2003; Hannes and Franck, 1983).

Despite these well known detrimental effects of social isolation, in animal experimentation individuals are commonly removed from their social groups and tested individually in standard experimental situations. Social isolation is also commonly used in behavioural endocrinological studies as a way to standardize the initial conditions (i.e. baseline hormone levels and previous social experience) in which animals enter an experiment (e.g. Galhardo et al., 2008; Oliveira et al., 1996, 2005). However, individual differences in the perception of social isolation are present in humans and modulate the above-mentioned impact of social isolation on biological function (e.g. Cacioppo et al., 2009; Cole et al., 2007). In recent years the concept that cognitive appraisal is involved in the processing of social information has been extended to animals (Mendl et al., 2010; Paul et al., 2005). Cognitive appraisal theory has been developed in emotion research and proposes that a response to an emotion-eliciting stimulus is not just a result of direct effects of perceptual information, but rather a function of what that perceptual information means to the organism at that particular time. Therefore, emotional experiences depend on some kind of general appraisal mechanism that allows organisms to evaluate the stimulus, that is its valence and salience, and to determine the appropriate

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physiological and behavioural response. Thus, the exactly same event, in the present case social isolation, may elicit different responses, depending on the way it is appraised by different individuals (e.g. dominants vs. subordinates) or by the same individual at different moments in time (e.g. in different social contexts).

In this study we investigate how social status and how previous social context modulate the androgen (i.e. testosterone, T and 11-ketotestosterone, KT) and cortisol response of male cichlid fish to social isolation. Cichlid fishes are excellent models for studying the effects of social factors on biological processes given their elaborate social behaviour repertoire, which is easily expressed in laboratory conditions (Baerends and Baerends-van Roon, 1950), and two species have been studied in detail in this respect: the Mozambique tilapia *Oreochromis mossambicus* and *Astatotilapia burtoni* (for recent reviews on each of these models please see Fernald, 2012; Oliveira, 2009, respectively). The Mozambique tilapia was chosen in this study given its robustness that allows for physiological manipulations (e.g. blood collection), and the high reward value given to conspecifics, which is similar to that of food reward (Galhardo et al., 2011). In this species territorial males have higher androgen levels than non-territorials, a difference that is driven by social status as it has been shown by an experiment in which individual androgen levels before group formation were not predictors of social status achieved after group formation, but rather social rank achieved was correlated with individual androgen levels after group formation (Oliveira et al., 1996). The increased androgen levels in males that become territorial reinforce their social status by increasing their chances to win subsequent interactions (androgen-mediated winner effect; Oliveira et al., 2009). Androgens also respond to social context in this species, namely to the exposure of third parties social interactions (i.e. bystander effect; Oliveira et al., 2001), in anticipation of a territorial intrusion (i.e. Pavlovian conditioning; Antunes and Oliveira, 2009), and as a function of the familiarity with the opponent (Aires et al., 2004). Interestingly, mirror elicited aggression experiments suggest that the androgen response to social challenge in this species is not simply determined by the exposure to the agonistic interaction, but rather by the animals' evaluation of the agonistic interaction (i.e. perceived win or perceived defeat; Oliveira et al., 2005). Thus, since androgen output seems not to be merely determined by social input, but rather by input evaluation, we hypothesized that the hormonal response to social isolation can as well differ between individuals depending on their evaluation of social isolation. Given that social isolation may be perceived either as a relief (in the case of non-territorials that hence avoid exposure to a dominant territorial male) or as a loss (in the case of territorial males that will loose their status), we predict a differential hormonal response to social isolation by males of different social status.

Materials and methods

Synopsis of the Mozambique tilapia social system

In nature *O. mossambicus* males form dense breeding aggregations in which each male defends a breeding territory centred in a spawning pit that is mouth-dug by the male (Bruton and Boltt, 1975). Territorial males exhibit a characteristic velvet-dark coloration, whereas non-territorial males and females exhibit a pale silver coloration (Baerends and Baerends-van Roon, 1950; Neil, 1964). Females visit male breeding aggregations when ready to spawn. Males court females and lead them to the spawning pits where spawning takes place (Baerends and Baerends-van Roon, 1950; Neil, 1964). Females collect the eggs into their mouths and leave to nursery areas in shallow waters where they mouthbrood the eggs and fry (Bruton and Boltt, 1975; Fryer and Iles, 1972). Non-territorial males are also present in the breeding aggregations and mimic females in order to gain access to spawning events in which they try to sneak fertilizations (Oliveira and Almada, 1998a). If a social opportunity arises non-territorial males can quickly become territorial, displaying the dark nuptial coloration and building a spawning pit (Oliveira and Almada, 1996, 1998b).

Fish housing and experimental procedures

Mozambique tilapia males from the stock held at ISPA (Oliveira et al., 1996) were used in this experiment. Fish were kept at a temperature of 26 °C ± 1 °C on a 12L:12D photoperiod. Social groups were composed by two males (focal animals) and three females per tank (M/F sex ratio = 0.67). Each tank (240 l glass aquarium, 120 × 40 × 50 cm) had a layer of fine gravel substrate. Tanks were supplied with a double filtering system (gravel and external biofilter, Eheim) and constant aeration. Water quality was analysed twice per month for nitrites (0.2–0.5 ppm), ammonia (<0.5 ppm) (Pallintest kit®) and pH (6.0–6.2). Fish were daily fed (in the morning) with 2% of their weight of commercial cichlid sticks (ASTRA). These social groups were left undisturbed for three weeks, in order to allow the formation of stable hierarchical structures (Oliveira and Almada, 1998a, 1998b). A total of 16 focal males (weight: 82 ± 5.3 g) were used in this study. Males' social status was identified prior to social isolation. Territorial males adopt a conspicuous nuptial black coloration and exhibit reproductive behaviour, including territory defence and digging of a spawning pit in the substrate. In 5 of the groups there was 1 territorial male and 1 non-territorial male. In the remaining 3 groups both males were territorial. After having been in these stable social groups, the focal males, previously classified as territorial or non-territorial males, were individually housed for one week, in aquaria (50 × 25 × 31 cm, 40 l) with opaque walls in order to visually isolate fish from outside stimuli. Therefore, during social isolation fish were deprived from any conspecific (visual, chemical, acoustic, or mechanic) stimuli. All other conditions remained the same as in the home tanks.

Blood sampling and hormone assays

Blood sampling for steroid hormone analysis took place on the last day of the 3 weeks of social grouping and on the 7th day of social isolation. At each sampling point fish were rapidly captured using a hand-net, and subsequently anaesthetised (stage two, Ross, 2001) in a solution of MS-222 (tricaine methane sulphonate, Sigma, St. Louis, MO, 200 ppm). Samples of 100–150 μl of blood were taken from the caudal vasculature (1 ml heparinised syringes; 25 G/16 mm needles). Fish were then placed in aerated water from 30 s to 1 min, in order for them to recover from anaesthesia. The entire process was performed in less than 4 min, which is below the reported latency for cortisol release into the systemic circulation in response to handling stress in this species (Foo and Lam, 1993). The non-conjugated, referred to as free, steroid fraction was extracted from the plasma by adding to the sample diethyl ether, as the steroid solvent. The samples were then centrifuged (5 min, 1000 rpm, 4 °C) and cooled for 10 min at −80 °C to separate the ether fraction, which remained liquid, from the frozen aquatic fraction. The steroids were isolated by evaporating the ether. This process was repeated twice.

All hormone levels were quantified by radioimmunoassay. Cortisol assays used the commercial antibody 'Anti-rabbit, Cortisol-3' [ref: 20-CR50, Interchim (Fitzgerald), Montluçon, France, cross-reactivity: cortisol 100%, prednisolone 36%, 11-Desoxycortisol 5.7%, Corticosterone 3.3%, Cortisone < 0.7%] and the radioactive marker [1,2,6,7-3H] Cortisol [ref: TRK407-250 mCi, Amersham Biosciences, NJ, USA]. Cortisol intra-assay variability was 5.8%. Levels of free KT fraction were determined using an antibody kindly donated by D.E. Kime (see Kime and Manning, 1982, for cross-reactivity and other antibody details). The T antibody (reference: RDITRK2T2) was purchased from Research Diagnostics Inc. (Concord, USA). T and KT intra-assay variability was 2.5% and 1.1% respectively.

Data analysis

In order to assess the moderator role of pre-isolation social context on the effect of social isolation on steroid hormone levels, two categorical variables were considered: (1) social status — territorial vs. non-territorial males; and (2) pre-isolation group composition — in some social groups there was a territorial male and a non-territorial male (TnT social group), whereas in others two territorial males co-existed (TT social group). Thus the potential effect of these two group types can only be tested for territorial males (i.e. territorial males from TT vs. territorial males TnT; non-territorials always come from the same group type, TnT). Given the formal lack of the non-territorial TT group, a two-way analysis (e.g. GLM) of these two categorical variables would render an incomplete design. Therefore, we further combined these two variables in order to classify socially isolated males into one of the following categories of 'previous social context': (1) territorial males from a TT group; (2) territorial males from a TnT group; or (3) non-territorial males from a TnT group. Since there was no correlation of the hormonal response to social isolation between males of the same group (cortisol: $R = 0.65$, $p = 0.08$; KT: $R = 0.12$, $p = 0.77$; T: $R = 0.15$, $p = 0.73$) these were treated as independent males in the analyses.

A repeated measures ANOVA was used to test the effect of social isolation (repeated measure: social group vs. social isolation) and of the combined variable previous social context (independent categorical variable: territorial TT vs. territorial TnT vs. non-territorial). Planned comparisons of least squares means were subsequently used to test the effects of social status (i.e. territorial TT + territorial TnT vs. non-territorial TnT) and of group composition (i.e. territorial TT vs. territorial TnT + non-territorial TnT) on the hormonal response to social isolation (i.e. social group vs. social isolation). Planned comparisons were also used to further test differences between specifc groups in the experiment namely:

(1) pre-isolation differences between different social status males (i.e. territorial TT + territorial TnT vs. non-territorial TnT only in social group);
(2) post-isolation differences between different social status males (i.e. territorial TT + territorial TnT vs. non-territorial TnT only in social isolation);
(3) within-social status effects of social isolation [i.e. (a) territorial TT + territorial TnT in social group vs. territorial TT + territorial TnT in social isolation; and (b) non-territorial TnT in social group vs. non-territorial TnT in social isolation];
(4) pre-isolation differences between different group types (i.e. territorial TT vs. territorial TnT + non-territorial TnT only in social group);
(5) post-isolation differences between different group types (i.e. territorial TT vs. territorial TnT + non-territorial TnT only in social isolation);
(6) within-group type effects of social isolation [i.e. (a) territorial TT vs. in social group vs. territorial TT in social isolation; and (b) territorial TnT + non-territorial TnT in social group vs. territorial TnT + non-territorial TnT in social isolation].

The assumptions of parametric statistics (normality and homogeneity of variances, assessed by Levene's test) were met and therefore non-transformed raw data was used in all tests. A p value of 0.05 was taken for significance in all statistical tests. The statistical package used for analysis was Statistica V.8® (StatSoft Inc., USA, 2008).

Ethical note

This experiment did not involve mortality of animals and all males were returned to their previous stock tanks after the experiments. The experimental procedures involved in these studies were in compliance with the regulations on animal experimentation in Portugal and were approved by a permit (Ref. 30489, 29/11/2007) from The Portuguese Veterinary Authorities (Direcção Geral de Veterinária, Portugal).

Results

There are no main effects of either 'previous social context' or social isolation on circulating T levels. There is also no interaction between

Table 1
Effects of social isolation, previous social group and social status on steroid hormone levels in male tilapia [repeated-measures ANOVA (repeated factor = before vs. after social isolation; categorical independent variable = pre-isolation social context) followed by planned comparisons of relevant variables]. Asterisks indicate significant differences: $^{*}p < 0.05$; $^{**}p < 0.01$; $^{***}p < 0.001$; marginally non-significant values (i.e. $0.05 < p < 0.10$) are indicated by §. Effect sizes are given as eta-squared values (η^2).

	Effects	Testosterone	11-Keto-testosterone	Cortisol
Main effects	Previous social context (PSC)	$F_{2,13} = 0.93$ $\eta^2 = 0.125$	$F_{2,13} = 3.61^{§}$ $\eta^2 = 0.357$	$F_{2,13} = 3.18^{§}$ $\eta^2 = 0.328$
	Social isolation (SI)	$F_{2,13} = 0.0001$ $\eta^2 = 0.000007$	$F_{2,13} = 1.57$ $\eta^2 = 0.068$	$F_{2,13} = 22.09^{***}$ $\eta^2 = 0.404$
Interaction	PSC × SI	$F_{1,13} = 1.69$ $\eta^2 = 0.207$	$F_{1,13} = 4.34^{*}$ $\eta^2 = 0.374$	$F_{1,13} = 9.83^{**}$ $\eta^2 = 0.359$
Planned comparisons	Social status (T vs. NT)	–	$F_{1,13} = 7.97^{*}$ $\eta^2 = 0.380$	$F_{1,13} = 5.72^{*}$ $\eta^2 = 0.305$
	Group type (TT vs. TnT)	–	$F_{1,13} = 4.39^{§}$ $\eta^2 = 0.252$	$F_{1,13} = 19.56^{***}$ $\eta^2 = 0.601$
	Territorial isolation ($T_{social\ group}$ vs. $T_{isolation}$)	–	$F_{1,13} = 7.38^{*}$ $\eta^2 = 0.362$	$F_{1,13} = 6.11^{*}$ $\eta^2 = 0.320$
	Non-territorial isolation ($NT_{social\ group}$ vs. $NT_{isolation}$)	–	$F_{1,13} = 2.47$ $\eta^2 = 0.159$	$F_{1,13} = 20.82^{***}$ $\eta^2 = 0.616$
	Social status in social group ($T_{social\ group}$ vs. $NT_{social\ group}$)	–	$F_{1,13} = 29.1^{***}$ $\eta^2 = 0.691$	$F_{1,13} = 0.11$ $\eta^2 = 0.009$
	Social status in isolation ($T_{isolation}$ vs. $NT_{isolation}$)	–	$F_{1,13} = 0.01$ $\eta^2 = 0.0008$	$F_{1,13} = 5.51^{*}$ $\eta^2 = 0.298$
	TT isolation ($TT_{social\ group}$ vs. $TT_{isolation}$)	–	$F_{1,13} = 6.43^{*}$ $\eta^2 = 0.331$	$F_{1,13} = 0.71$ $\eta^2 = 0.052$
	TnT isolation ($TnT_{social\ group}$ vs. $TnT_{isolation}$)	–	$F_{1,13} = 0.02$ $\eta^2 = 0.002$	$F_{1,13} = 37.66^{***}$ $\eta^2 = 0.743$
	Group type in social group ($TT_{social\ group}$ vs. $TnT_{social\ group}$)	–	$F_{1,13} = 10.07^{**}$ $\eta^2 = 0.436$	$F_{1,13} = 1.31$ $\eta^2 = 0.092$
	Group type in isolation ($TT_{isolation}$ vs. $TnT_{isolation}$)	–	$F_{1,13} = 0.322$ $\eta^2 = 0.024$	$F_{1,13} = 15.97^{*}$ $\eta^2 = 0.551$

'previous social context' and social isolation (Table 1, Fig. 1A). Therefore, no further statistical analyses were performed for this androgen. The main effect of 'previous social context' is marginally non-significant both for KT and for cortisol (Table 1). The main effect of social isolation is not significant for KT (Table 1, Fig. 1B) but it is significant for cortisol (social group < social isolation, Table 1, Fig. 1C). There is a significant interaction between 'previous social context' and social isolation both for KT and for cortisol (Table 1). Therefore, subsequent planned comparison analyses were performed to disentangle the putative moderator roles of the raw variables (i.e. social status and group composition) that build up the composite variable 'previous social context'.

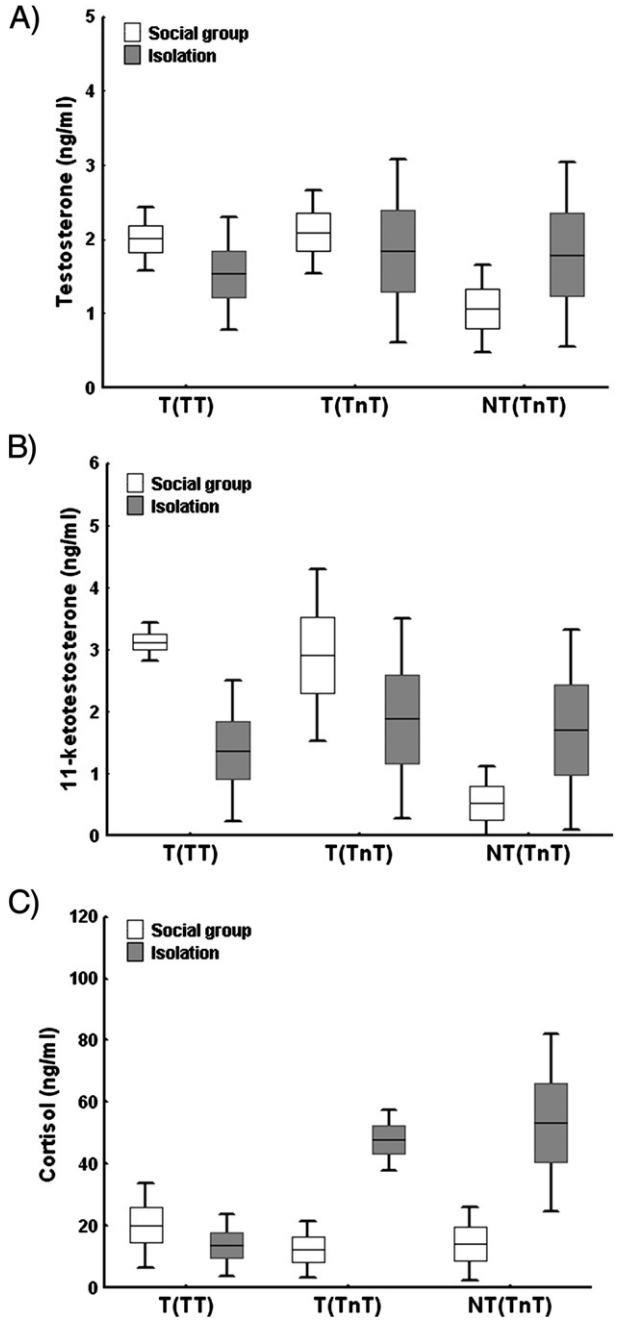

Fig. 1. Effect of social isolation on (A), testosterone (B) 11-ketotestosterone and (C) cortisol levels. In the box-and-whisker plots the middle point represents the mean, the box represents the standard error of the mean, and the whiskers represent the standard deviation; T(TT) = territorial males from social groups with 2 territorials; T(TnT) = territorial males from social groups with 1 territorial and 1 non-territorial; NT(TnT) = non-territorial males from social groups with 1 territorial and 1 non-territorial.

Moderator role of social status on the effects of social isolation on steroid levels

Social status (territorial vs. non-territorial) has a moderator role on the effect of social isolation both on KT and cortisol levels (Table 1).

Social isolation induces a reduction in KT in territorial males but not in non-territorials (Table 1), in which a trend for an increase in KT levels in social isolation is observed (Fig. 1B). Therefore, the difference in KT levels between territorial and non-territorial individuals observed in the pre-isolation social group is no longer present under social isolation (Table 1, Fig. 1B).

Social isolation induces a significant increase in cortisol levels both in territorial and non-territorial males (Table 1, Fig. 1C). However, the increase is higher in non-territorial males such that there are no differences in cortisol levels between the two male types in social groups, but non-territorials have higher cortisol levels in social isolation (Table 1, Fig. 1C).

Moderator role of pre-isolation group composition on the effects of social isolation on steroid levels

Since social groups varied with 2 territorial males present in some tanks and a territorial and a non-territorial male present in others, we decided to further analyse the moderator effect of pre-isolation group composition (i.e. TT = 2 territorial males vs. TnT = 1 territorial male and 1 non-territorial male) on social isolation for territorial males (see Fig. 1). We did not perform this analysis for non-territorial males given that by definition all non-territorial males lived in a territorial +non-territorial social group. Pre-isolation group composition (TT vs. TnT) modulates the effect of social isolation both on KT and on cortisol levels (Table 1), but with mirroring effects. TT territorial males, but not TnT territorial males, decrease KT levels in social isolation, such that differences in KT levels between these two male types that are present in pre-isolation social groups disappear after social isolation (Table 1). Conversely, TnT territorial males increase cortisol levels in social isolation, but TT territorial males maintain similar levels, such that a significant difference between territorial TT and TnT male cortisol levels is present in social isolation but not in pre-isolation social groups (Table 1, Fig. 1C).

Discussion

In this study we show that the effects of social isolation on steroid hormone levels are modulated by previous social status and by social group composition before isolation.

In social conditions, androgens (KT) were higher in territorial males than in non-territorials, as has already been shown for this species (Oliveira et al., 1996). This difference between territorial and non-territorial males in androgen levels disappears after 7 days of social isolation, suggesting that, as suggested in previous studies (Galhardo et al., 2008; Oliveira et al., 1996, 2005), social isolation is an efficient way to standardize initial androgen levels in experimental studies. However, social isolation elicits opposite KT responses in males of different social status, such that socially isolated territorial males decrease their KT levels whereas non-territorial males present a non-significant trend for increasing KT levels (or following a more conservative interpretation, at least do not exhibit a decrease). Previous studies on the effects of social isolation on fish androgen levels have produced conflicting results. In the cichlid (*A. burtoni*) and in swordtail fish (*Xiphophorus helleri*), 4 to 8 weeks of social isolation induced a general reduction in androgen levels (Hannes and Franck, 1983), whereas in two hybridizing cichlid species from Lake Victoria (*Pundamilia nyererei* and *Pundamilia pundamilia*) it had no effect (Dijkstra et al., 2011). However, social status was not taken into account in these studies, which could have explained the divergent results. The differential androgen response to social isolation between territorial and non-territorial males also supports the view expressed elsewhere (Oliveira, 2009; Oliveira and Canario, 2011; Oliveira et al., 2005) that the triggering of the androgen response to the social environment depends on the evaluation of the event by each specific organism in that

moment in time (i.e. cognitive appraisal, sensu Paul et al., 2005), rather than on the objective structure of the situation. In this case, the change from living in a social group to social isolation would have been perceived as losing status by territorial males, whereas non-territorials would have perceived it as an opportunity to gain status (or at least as release from social subordination). Interestingly, pre-isolation group composition also modulates the androgen response to social isolation, such that among territorial males only males from TT groups decreased their KT levels in isolation. This result suggests that the shift from a social condition to isolation is perceived differently between TT and TnT territorial males, hence giving further support to the appraisal hypothesis presented above (e.g. Paul et al., 2005).

Under stable social conditions males of both social status showed similar levels of cortisol, but after social isolation cortisol increased significantly only in individuals that came from an asymmetric social group (i.e. TnT social groups), independent of social status. That is, cortisol increases in both territorials and non-territorials coming from TnT social groups, but not in territorials coming from TT groups. Other studies have also reported similar levels of cortisol between territorial and non-territorial tilapia males under social conditions (Correa et al., 2003; Galhardo et al., 2008) and increased cortisol in social isolation has been reported for others fish species (e.g. Earley et al., 2006; although in other cichlid species cortisol levels have been reported to be lower than in a social condition, Dijkstra et al., 2011). The fact that the cortisol response to social isolation is only present in males coming from an asymmetric social group, composed by a territorial male and a non-territorial male, is puzzling. Differences in response to social isolation between males of different social status have already been reported in fish (Gómez-Laplaza and Morgan, 2000, 2003), but to the best of our knowledge this is the first report of an effect of previous social grouping irrespective of social status. A possible explanation for this result, that requires further experimental testing, is that social isolation is not being perceived as a stressor by males from a social group composed by 2 territorial males, in which threat perception must be higher than by either territorial and non-territorial males in an asymmetric social group, where social ranks are well established.

In summary, here we show that isolation-induced steroid hormone responses are modulated by social factors, with the androgen (KT) response being influenced by previous social status and the cortisol response being influenced by previous social grouping. These results highlight the problem that may arise in behavioural endocrinological research when using social isolation in experimental designs as a way of standardising the initial conditions of subjects.

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References

Aires, R.F., Ros, A.F.H., Oliveira, T., Oliveira, R.F., 2004. Androgens and the dear enemy effect in a cichlid fish. Horm. Behav. 46, 106.
Al-Imari, L., Gerlai, R., 2008. Sight of conspecifi cs as reward in associative learning in zebrafish (*Danio rerio*). Behav. Brain Res. 189, 216–219.
Antunes, R.A., Oliveira, R.F., 2009. Hormonal anticipation of territorial challenges in cichlid fish. Proc. Natl. Acad. Sci. 106, 15985–15989.
Baerends, G.P., Baerends-van Roon, J.M., 1950. An introduction to the study of the ethology of the cichlid fishes. Behav. Suppl. 1–243.
Bruton, M., Boltt, R., 1975. Aspects of the biology of *Tilapia mossambica* Peters (Pisces: Cichlidae) in a natural fresh-water lake (Lake Sibaya, South Africa). J. Fish Biol. 7, 423–445.
Cacioppo, J.T., Norris, C.J., Decety, J., Monteleone, G., Nusbaum, H., 2009. In the eye of the beholder: individual differences in perceived social isolation predict regional brain activation to social stimuli. J. Cogn. Neurosci. 21, 83–92.
Cacioppo, J.T., Hawkley, L.C., Norman, G.J., Berntson, G.G., 2011. Social isolation. Ann. N. Y. Acad. Sci. 1231, 17–22.
Cole, S.W., Hawkley, L.C., Arevalo, J.M., Sung, C.Y., Rose, R.M., Cacioppo, J.T., 2007. Social regulation of gene expression in human leukocytes. Gen. Biol. 8, R189.
Correa, S.A., Fernandes, M.O., Iseki, K.K., Negrao, J.A., 2003. Effect of the establishment of dominance relationships on cortisol and other metabolic parameters in Nile tilapia (*Oreochromis niloticus*). Braz. J. Med. Biol. Res. 36, 1725–1731.
Dijkstra, P.D., Wiegertjes, G.F., Forlenza, M., van der Sluijs, I., Hofmann, H.A., Metcalfe, N.B., Groothuis, T.G., 2011. The role of physiology in the divergence of two incipient cichlid species. J. Evol. Biol. 24, 2639–2652.
Earley, R.L., Edwards, J.T., Aseem, O., Felton, K., Blumer, L.S., Karom, M., Grober, M.S., 2006. Social interactions tune aggression and stress responsiveness in a territorial cichlid fish (*Archocentrus nigrofasciatus*). Physiol. Behav. 88, 353–363.
Fernald, R.D., 2012. Social control of the brain. Annu. Rev. Neurosci. 35, 133–151.
Fernald, R.D., Maruska, K.P., 2012. Social information changes the brain. Proc. Natl. Acad. Sci. U. S. A. 109 (Suppl. 2), 17194–17199.
Foo, J.T.W., Lam, T.J., 1993. Serum cortisol response to handling stress and the effect of cortisol implantation on testosterone level in the tilapia, *Oreochromis mossambicus*. Aquaculture 115, 145–158.
Fryer, G., Iles, T.D., 1972. The Cichlid Fishes of the Great Lakes of Africa: Their Biology and Evolution. Oliver & Boyd, Edinburgh, U.K.
Galhardo, L., Correia, J., Oliveira, R.F., 2008. The effect of substrate availability on behavioural and physiological indicators of welfare in the African cichlid (*Oreochromis mossambicus*). Anim. Welf. 17, 239–254.
Galhardo, L., Almeida, O., Oliveira, R.F., 2011. Measuring motivation in a cichlid fish: an adaptation of the push-door paradigm. Appl. Anim. Behav. Sci. 130, 60–70.
Gómez-Laplaza, L.M., Morgan, E., 2000. Laboratory studies of the effects of short-term isolation on aggressive behaviour in fish. Mar. Freshwater Behav. Physiol. 33, 63–102.
Gómez-Laplaza, L.M., Morgan, E., 2003. The influence of social rank in the angelfish, *Pterophyllum scalare*, on locomotor and feeding activities in a novel environment. Lab. Anim. 37, 108–120.
Hannes, R.-P., Franck, D., 1983. The effect of social isolation on androgen and corticosteroid levels in a cichlid fish (*Haplochromis burtoni*) and in swordtails (*Xiphophorus helleri*). Horm. Behav. 17, 292–301.
Hennessy, M.B., 1997. Hypothalamic–pituitary–adrenal responses to brief social separation. Neurosci. Biobehav. Rev. 21, 11–29.
Kime, D.E., Manning, N.J., 1982. Seasonal patterns of free and conjugated androgens in the brown trout *Salmo trutta*. Gen. Comp. Endocrinol. 48, 222–231.
Mendl, M., Burman, O.H., Paul, E.S., 2010. An integrative and functional framework for the study of animal emotion and mood. Proc. R. Soc. B 277, 2895–2904.
Neil, E., 1964. An analysis of colour changes and social behaviour of *Tilapia mossambica*. Univ. Calif. Publ. Zool. 75, 1–58.
Oliveira, R.F., 2009. Social behavior in context: hormonal modulation of behavioral plasticity and social competence. Integr. Comp. Biol. 49, 423–440.
Oliveira, R.F., Almada, V.C., 1996. On the (in)stability of dominance hierarchies in the cichlid fish *Oreochromis mossambicus*. Aggress. Behav. 22, 37–45.
Oliveira, R.F., Almada, V.C., 1998a. Mating tactics and male–male courtship in the lek-breeding cichlid *Oreochromis mossambicus*. J. Fish Biol. 52, 1115–1129.
Oliveira, R.F., Almada, V.C., 1998b. Dynamics of social interactions during group formation in males of the cichlid fish *Oreochromis mossambicus*. Acta Ethol. 1, 57–70.
Oliveira, R.F., Canario, A.V., 2011. Nemo through the looking-glass: a commentary on Desjardins & Fernald. Biol. Lett. 7, 487–488.
Oliveira, R.F., Almada, V.C., Canario, A.V.M., 1996. Social modulation of sex steroid concentrations in the urine of male cichlid fish *Oreochromis mossambicus*. Horm. Behav. 30, 2–12.
Oliveira, R.F., Lopes, M., Carneiro, L.A., Canario, A.V.M., 2001. Watching fights raises fish hormone levels. Nature 409, 475.
Oliveira, R.F., Carneiro, L.A., Canario, A.V.M., 2005. No hormonal response in tied fights. Nature 437, 207–208.
Oliveira, R.F., Silva, A., Canario, A.V.M., 2009. Why do winners keep winning? Androgen mediation of winner but not loser effects in cichlid fish. Proc. R. Soc. Lond. B 276, 2249–2256.
Paul, E.S., Harding, E.J., Mendl, M., 2005. Measuring emotional processes in animals: the utility of a cognitive approach. Neurosci. Biobehav. Rev. 29, 469–491.
Ross, L.G., 2001. Restraint, anaesthesia & euthanasia, In: Wildgoose, W.H. (Ed.), Veterinary Practice & Procedures for Ornamental Fish, 2nd ed. British Small Animal Veterinary Association, London, UK, pp. 75–83.
Ruan, H., Wu, C.F., 2008. Social interaction-mediated lifespan extension of Drosophila Cu/Zn superoxide dismutase mutants. Proc. Natl. Acad. Sci. 105 (21), 7506–7510.
Thiel, K.J., Okun, A.C., Neisewander, J.L., 2008. Social reward-conditioned place preference: a model revealing an interaction between cocaine and social context rewards in rats. Drug Alcohol Depend. 96, 202–212.