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Androgen response to social competition in a shoaling fish

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ABSTRACT

Androgens respond to social challenges and this response has been interpreted as a way for males to adjust androgen-dependent behavior to social context. However, the androgen responsiveness to social challenges varies across species and a conceptual framework has been developed to explain this variation according to differences in the mating system and parental care type, which determines the regimen of challenges males are exposed to, and concomitantly the scope (defined as the difference between the physiological maximum and the baseline levels) of response to a social challenge. However, this framework has been focused on territorial species and no clear predictions have been made to gregarious species (e.g. shoaling fish), which although tolerating same-sex individuals may also exhibit intra-sexual competition. In this paper we extend the scope of this conceptual framework to shoaling fish by studying the endocrine response of zebrafish (*Danio rerio*) to social challenges. Male zebrafish exposed to real opponent agonistic interactions exhibited an increase in androgen levels (11-ketotestosterone both in Winners and Losers and testosterone in Losers). This response was absent in Mirror-fighters, that expressed similar levels of aggressive behavior to those of winners, suggesting that this response is not a mere reflex of heightened aggressive motivation. Cortisol levels were also measured and indicated an activation of the hypothalamic–pituitary–interrenal axis in Winners of real opponent fighters, but not Losers or in Mirror-fighters. These results confirm that gregarious species also exhibit an endocrine response to an acute social challenge.

Introduction

An androgen response to social challenge has been described across vertebrates, from fish to humans (Archer, 2006; Oliveira et al., 2002). This response has been interpreted as a way for individuals to adjust their expression of androgen-dependent behaviors to social context (Oliveira, 2009). However, these socially driven changes in androgen levels vary widely across species (Hirschenhauser and Oliveira, 2006). The “Challenge Hypothesis” has been proposed to explain this variation based on inter-specific differences in the exposure to social challenges due to variation in mating system and/or parental care type (Wingfield et al., 1990; for reviews more recent reviews see Goymann et al., 2007; Hirschenhauser and Oliveira, 2006). For example, males from monogamous species with paternal care are expected to experience a lower regimen of intra-sexual competition than males from polygamous species without paternal care, and therefore the former are expected to have lower breeding baseline androgen levels and concomitantly a higher scope of response (i.e. the potential increase from baseline levels up to the physiological maximum) when faced with a social challenge. These predictions of the “Challenge Hypothesis” have been in general confirmed across different taxa, but most studies so far have concentrated on territorial species (e.g. fish, Hirschenhauser et al., 2004; birds, Hirschenhauser et al., 2003). However, gregarious species present an interesting case for testing the “Challenge Hypothesis” since they have a high tolerance for the presence of same-sex conspecifics, which in territorial species would be treated as intruders, but still compete for the access to mating opportunities. Thus, social baseline androgen levels are expected to be low, enabling a high scope of response to social challenges.

Shoaling fish have been mainly studied from a perspective of affiliative behavior (e.g. Engeszer et al., 2004; Lachlan et al., 1998). However, shoaling species also exhibit structured social relationships with social hierarchies and leader–follower roles (e.g. zebrafish, Paull et al., 2010; Vital and Martins, 2013), which require some kind of agonistic regulation. Thus, aggressive behavior is also relatively common in shoaling species and it increases in mating contexts, being modulated both by operational sex-ratio and by density (e.g. zebrafish, Spence and Smith, 2005). Moreover, both males and females of shoaling species may express aggressive behavior when competing for resources (e.g. zebrafish, Paull et al., 2010). The relationship between androgens and social status in shoaling species varies, with dominant individuals having higher androgen levels than subordinates in some species (e.g. zebrafish, Filby et al., 2010) but not in others (e.g. swordtail fish,

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Hannes, 1984). Therefore, the associations found between androgen levels and social status in male shoaling teleosts may be explained by the challenge hypothesis, reflecting a higher regimen of social challenges in dominant males (Oliveira et al., 2002).

In this study we tested the hypothesis that an acute social challenge elicits an androgen response in a shoaling species, the zebrafish (*Danio rerio*), by promoting dyadic interactions between males. Furthermore, we also tested the hypothesis that it is the perception of the outcome of the interaction as a victory or a defeat that triggers the androgen response, rather than the mere expression of aggressive behavior. For this purpose we had a treatment with mirror-elicited aggression, where males fought their own image on a mirror. In zebrafish, mirror fights elicit similar levels of aggressive behavior to those observed in real opponent fights (Teles et al., 2013) and since the behavior expressed by the mirror-image matches the behavior of the focal individual, no information on fight outcome is available (Oliveira et al., 2005). Thus, despite expressing similar levels of aggressive behavior to those of winners of real opponent fights, Mirror fighters do not experience either a victory or a defeat. Thus, if the androgen response depends on the perception of fight outcome we predict androgen levels to increase in real opponent fighters but not in Mirror-fighters. Since social challenges may also activate the hypothalamic–pituitary–interrenal (HPI) axis we have also sampled cortisol.

Material and methods

Animal housing

Adult zebrafish males from the AB strain bred and held at Instituto Gulbenkian de Ciência (IGC, Oeiras, Portugal) were used in this study. Fish were kept in a recirculating system (ZebraTec 93, Tecniplast), at 28 °C with a 14L:10D photoperiod. Water system was monitored for nitrites (<0.2 ppm), nitrates (<50 ppm) and ammonia (0.01–0.1 ppm), and pH and conductivity maintained at 7 and 700 μSm respectively. Fish were fed twice a day with *Artemia salina* in the morning and commercial food flakes in the afternoon, except on the day of the experiments.

Social challenge tests

Four social treatments were used in this study ($N = 16$ for each treatment): (1) fish that won a social interaction against a real opponent (Winners); (2) fish that lost a social interaction against a real opponent (Losers); (3) fish that interacted with its own image on a mirror and that despite fighting neither won nor lost the social interaction (Mirror-fighters); and (4) fish that did not experience a social interaction, which was used as a reference group (Control). The real opponent interactions followed a previously described behavioral paradigm (Oliveira et al., 2011). In brief, each pair was placed in the experimental arena (5 cm × 8 cm × 6 cm) where they stayed overnight isolated from each other by a removable opaque PVC partition. On the next day, the opaque divider was removed and the fish allowed to interact for 30 min. The mirror-elicited interactions followed a similar procedure but at the time of the interaction, the opaque partition separating the opponents remained in place, and a pair of mirrors was revealed, one on each compartment. Therefore, pairs of Mirror-fighters fought their own image independently but simultaneously. This procedure allowed control for the presence of putative chemical cues during agonistic interactions, since the two compartments were not isolated chemically from each other. Pairs from the control treatment also stayed in the same conditions of the other experimental treatments but at the time of the interaction the opaque partition was not lifted and they remained in visual isolation from each other. Fish were matched for size across treatments [body weight (mean ± SEM) = 0.32 ± 0.01 g; standard length (mean ± SEM) = 2.78 ± 0.006 cm]. Behavioral tests were video-recorded for subsequent behavioral analysis.

Hormone assays

Due to the small size of zebrafish, blood collection is difficult and provides small volumes of plasma. Therefore, in this study we have assayed steroid hormones from whole-body homogenates rather than from plasma. Immediately after the interaction fish were killed using an overdose of anesthetic (MS222, Pharmaq; 500–1000 mg/L) followed by spinal cord sectioning. Whole-body samples were collected and frozen at −80 °C. Steroid extraction from whole-body samples followed the protocol described in Cachat et al., 2010. Whole-body concentrations of 11-ketotestosterone (KT), testosterone and cortisol were measured using specific enzyme immunoassay kits (Cayman Chemical Company #582751, #582701, #500360, respectively) following the manufacturer's instructions. All Samples were tested in duplicate in a dilution of, 1:40 for the KT, 1:20 for testosterone and 1:4 for cortisol. Intra-assay and inter-assay coefficients of variation were respectively: 4.2%, and 12.8% for KT, 1.7% and 5.1% for testosterone, and 6.6% and 14.6% for cortisol.

Behavioral analysis

Behavioral analysis was performed using a computerized multi-event recorder (Observer XT, Noldus, Wageningen, The Netherlands). As previously described (Oliveira et al., 2011), dyadic male fights in zebrafish are composed by two distinct phases: (1) the pre-resolution phase, where the interaction is symmetric and both fish exhibit the same repertoire of behaviors (i.e. display, circle, and bite); and (2) the post-resolution phase, where all aggressive behaviors are initiated by the Winner and the Loser only displays submissive behaviors. Here we only analyzed the last 5 min of the interaction, corresponding to the post-resolution phase, when dominance relationships have already been established and Winners and Losers express status-specific behavioral profiles. Thus aggressive behaviors (i.e. chase, bite, strike) were only scored for Winners and submissive behaviors (i.e. flee, freeze) for Losers, following the available zebrafish ethogram (Oliveira et al., 2011). Aggressive and submissive behaviors where then combine into two variables (aggression and submission) for subsequent behavioral analysis since a PCA performed for the same variables in the same behavioral paradigm clearly separated aggressive and submissive behaviors into independent factors (Teles et al., 2013).

Data analysis

Behavioral data did not conform to parametric assumptions, hence the comparison of aggressive behavior between Winners and Mirror-fighters was performed using the non-parametric Mann–Whitney U test. Hormonal data was log transformed to meet parametric criteria, which were checked by values of skewness and kurtosis for normality, and by the Levene test for the homogeneity of variances. A one-way ANOVA was used to test the main effect of social treatment on hormone levels, followed by Tukey HSD post-hoc tests to assess differences between each of the social treatments. Effect sizes were computed for all tests [eta-squared (η^2) for ANOVA and Cohen d_s for post-hoc tests]. Pearson correlations were used to assess the association between hormone levels and behavior expression. Sample sizes varied between groups due to outlier values (i.e. mean ± 3 × standard deviation). All statistical tests were two-tailed with a p-level of 0.05, and were performed using the software STATISTICA v.10.

Ethics statement

All procedures used in this study followed the institutional guidelines for the use of animals in experimentation and were approved both by the internal Ethics Committee of the Gulbenkian Institute of Science and by the National Veterinary Authority (Direção Geral de Alimentação e Veterinária, Portugal; permit number 8954).

Results

Behavioral analysis confirmed that dominance relationships were established in real opponent fights with Winners only exhibiting aggressive behavior, and Losers only expressing submissive behavior after the fight resolution (Fig. 1A). Mirror-fighters only expressed aggressive behavior at a frequency that was not significantly different from that of Winners from the real opponent interaction (Mann–Whitney test: $Z = 0.642$, $p = 0.52$, Fig. 1A).

There were significant main effects of social treatment on whole-body levels for all measured hormones (KT: $F_{3,\,53} = 4.260$, $p = 0.009$, $\eta^2 = 0.194$; testosterone: $F_{3,\,57} = 2.946$, $p = 0.040$, $\eta^2 = 0.134$; cortisol: $F_{3,\,55} = 4.112$, $p = 0.011$, $\eta^2 = 0.183$). Post-hoc analysis revealed that for the KT both Winners and Losers had higher levels than the Control group ($p = 0.0099$, $d_s = 1.051$; and $p = 0.047$, $d_s = 0.842$; respectively; Fig. 1C). For testosterone, the post-hoc tests detected higher levels in Losers than in Controls ($p = 0.048$, $d_s = 0.834$) and a close to significant difference with Mirror fighters ($p = 0.08$, $d_s = 0.767$; Fig. 1B). Finally, the post-hoc analyses for cortisol revealed that Winners had higher cortisol levels than Controls ($p = 0.023$, $d_s = 1.409$), and close to significant higher levels than Mirror-fighters ($p = 0.06$, $d_s = 0.963$; Fig. 1D).

Correlation analysis between behavior and hormone levels only revealed a single positive correlation between cortisol and the expression of aggressive behavior in the mirror group ($r = 0.747$, $n = 12$, $p = 0.005$). Correlations between hormones within each social treatment were also computed, but there was only one significant positive correlation between testosterone and KT for Losers ($r = 0.564$, $n = 15$, $p = 0.029$). All other correlations were non significant.

Discussion

Overall the results presented in this paper confirmed the occurrence of an androgen response to a social challenge in zebrafish. Indeed, KT levels increased in real opponent fighters in comparison to Controls, irrespective of the social status achieved (i.e. both in Winners and in Losers), whereas testosterone levels only increased in Losers, which exhibited higher levels than either Controls or Mirror-fighters. However, these results did not confirm the prediction that Winners would increase and Losers decrease their androgen levels, as a way to adjust androgen-dependent behavior to perceived social status (Oliveira, 2009). Thus, KT seems to be responding to the fact that the agonistic interaction has been solved, rather than to the specific outcome (i.e. winning or losing). Alternatively, the KT increase in Losers, which is the result that contradicts the prediction, may reflect some resilience of Losers to a descent in social status after a single interaction. Further experiments are needed to disentangle these two possibilities. On the other hand, the increase of testosterone levels only in Losers may reflect the fact that testosterone can be metabolized into KT, and that one of the possible physiological consequences of subordinance may be the blockage of KT production, resulting in an accumulation of the precursor testosterone. Indeed, in Siamese fighting fish (*Betta splendens*) subordinate males, which have a lower expression of aggressive behavior than dominants, have the enzyme 11-beta-hydroxylase blocked (Leitz, 1987). This is also consistent with the view that KT is the main androgen in the regulation of male behavior among teleosts (Borg, 1994; Gonçalves and Oliveira, 2010; Oliveira and Gonçalves, 2008). The results presented here also contrast with previously reported differences in KT levels between dominant and subordinate fish in long-term interactions

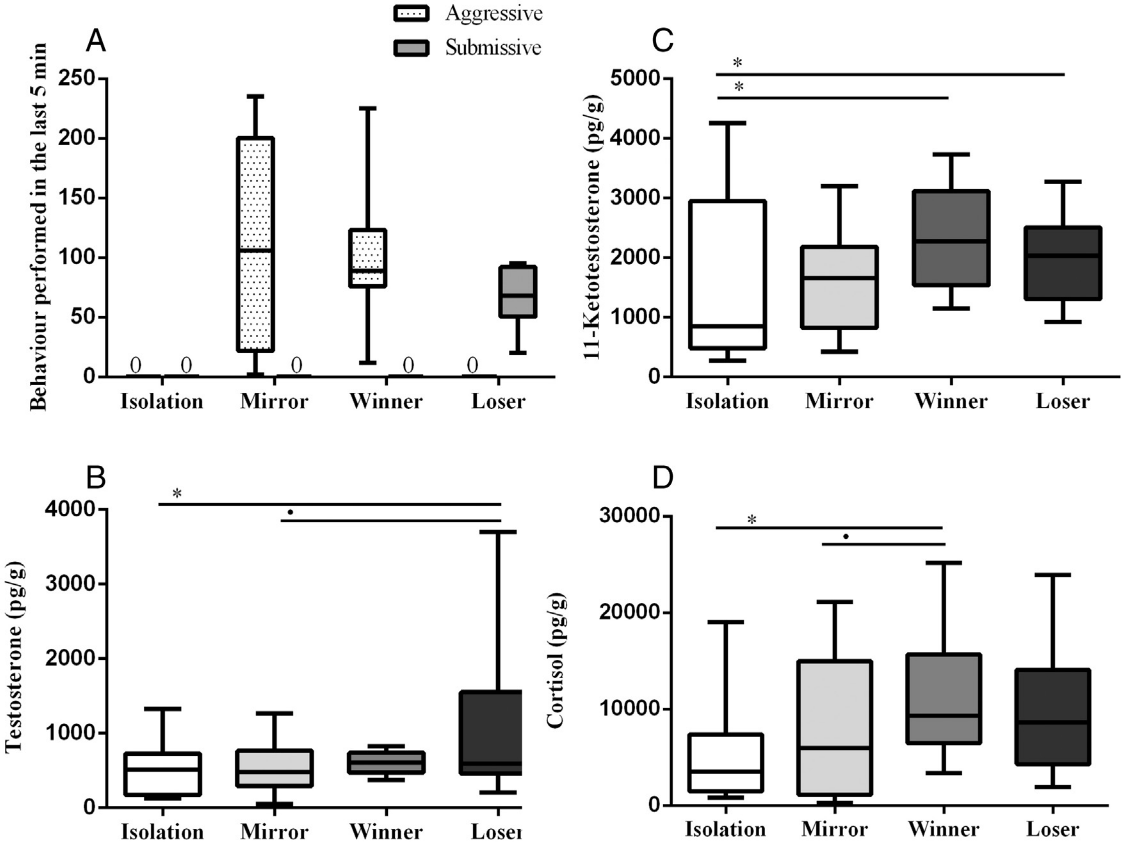

Fig. 1. Effects of social challenge on agonistic behavior and hormonal levels: (A) Behavioral characterization of the social treatments, as the frequency of aggressive and submissive behaviors expressed at the last 5 min of the agonistic interactions (sample sizes: Isolation = 16, Mirror fighters = 15, Winners = 15, Losers = 14); (B) whole-body testosterone levels (sample sizes: Isolation = 15, Mirror fighters = 15, Winners = 15, Losers = 16); (C) whole-body 11-ketotestosterone levels (sample sizes: Isolation = 14, Mirror fighters = 14, Winners = 14, Losers = 15); (D) whole-body cortisol levels (sample sizes: Isolation = 13, Mirror fighters = 14, Winners = 16, Losers = 16). A standard boxplot is presented with a dark line representing the median, the box representing the lower quartile and the upper quartiles, and the whiskers representing the minimum and maximum values. Asterisks indicate significant differences between treatments ($p < 0.05$), and dots indicate close to significant differences ($0.05 < p < 0.10$).

(i.e. individuals paired for either 1 or 5 days) (Filby et al., 2010). This difference between the two studies can be explained at least in two different ways: (1) in our study the individuals were sampled immediately after the social challenge, which may have missed a divergent time course in the androgen response between Winners and Losers that was captured when individuals were sampled after 1 or 5 days; and/or (2) the cumulative asymmetry of social status may elicit divergent androgen profiles between dominants and subordinates, which are not present immediately after a first interaction but that emerge with continuous interactions.

Despite the similar levels of aggressive behavior expressed by Mirror-fighters and Winners of real opponent interactions, Mirror-fighters did not exhibit an androgen response (i.e. had similar levels of both KT and testosterone to those of non-interacting Controls). Together these results indicate a decoupling between the expression of aggressive behavior and the androgen response to social challenge in zebrafish, which cannot be explained merely as a reflex of a heightened aggressive motivation. This result is in line with previous studies in other species, which have also reported a dissociation between the androgen and the behavioral response in mirror-elicited aggression (Hirschenhauser et al., 2008; Oliveira et al., 2005). However, it should be noted here that some other studies have found an androgen response to mirror elicited fights (Desjardins and Fernald, 2010; Dijkstra et al., 2012). The specific factor that signals fight outcome and that is absent in Mirror-fights in some studies but not in others is difficult to identify (Oliveira and Canário, 2011). When compared to real opponent fights, mirror-elicited fights lack some components that may be relevant for the participants to assess their relative fighting ability, namely: (1) the possibility to assume an antiparallel (i.e. head to tail) position and to circle the opponent; (2) the initiative (of the mirror image) to display a different behavior from that expressed by the opponent (i.e. focal fish); (3) putative hydrodynamic signals produced by the opponent and sensed by the lateral line; and/or (4) potential physical injuries in escalated phases of the fight (for detailed discussions of these aspects see: Arnott et al., 2011; Elwood et al., 2014). However, it has been shown recently that visual information alone is sufficient for zebrafish to assess the social status of conspecifics and to adjust their behavior accordingly (Abril-de-Abreu et al., 2015), and thus the visual information available in mirror-fights should be enough to convey the relevant information for dominance assessment. Independently of the relative importance of one or more of the above factors for fight resolution and for the triggering of the androgen response, the end result was that mirror-fights were not resolved in the present study (i.e. the focal fish never gave up fighting), despite the fact that the duration of the behavioral trials used here was much above the time it typically takes for a dominant–subordinate relationship to be established in a pair of real opponents (Oliveira et al., 2011).

Although the experimental paradigm used in this study used staged dyadic interactions in the absence of other conspecifics, in nature fights occur most probably in the presence of an audience. We have recently shown that when fighting in the presence of conspecifics male zebrafish increase the displaying phase of the fights that take longer to be resolved (Cruz and Oliveira, 2015). Thus, it is expected that the androgen levels measured here are an underestimation of those that would be observed in the presence of an audience. Indeed, in another teleost species (Siamese fighting fish, *B. splendens*) KT levels in real opponent fighters are much higher when the interaction occurs in the presence of a male audience (Dzieweczynski et al., 2006).

Given the gregarious nature of zebrafish it could be predicted that the isolation condition, used here as a control, would increase stress and HPI axis activity in relation to the social treatments. Although this is a reasonable prediction that has been confirmed in other social teleosts (e.g. cichlids, Galhardo and Oliveira, 2014), it should be noted that in this study we did not compare isolated fish with shoaling fish, but rather with fish engaged in fighting interactions after being socially isolated. Thus, our social treatments (i.e. fights) are predicted to elicit a higher stress response than that observed in the isolated control fish. Interestingly, the measures of cortisol taken to assess social stress during social challenges indicate that only Winners of real opponent fights have increased cortisol when compared either to controls or to Mirror-fighters. Both Losers of real opponent fights and Mirror-fighters also increased their cortisol levels but these were not higher than the levels observed in socially isolated fish. Thus, in acute social interactions Winners seem to incur in higher social stress than Losers. This result contrasts with the higher cortisol levels observed in both dominant and subordinate individuals of long-term interactions (5 days), when compared to control levels in non-interacting fish (Pavlidis et al., 2011). This difference between the cortisol response to acute vs. chronic social challenges may reflect an earlier stress response experienced by dominants during hierarchy formation, that only later on is also experienced by losers. If this is the case it represents a metabolic cost for the establishment of social status that may have fitness consequences.

Due to its small size zebrafish provides very small amounts of plasma (ca. 5 μl of plasma are collected from a 2.5 cm standard length fish). Thus, in this study we have assayed steroid hormone levels from whole-body homogenates. This is a common procedure when assaying hormones in small fish and it has been used previously in zebrafish to measure cortisol responses to stressors (Barcellos et al., 2007; Cachat et al., 2010; Fuzzen et al., 2010; Pottinger and Calder, 1995; Ramsay et al., 2006, 2009). Interestingly, whole-body cortisol measures respond not only to chronic but also to acute stressors, hence suggesting that whole-body steroid measures capture transient changes in steroid levels. Therefore, despite the fact that the “Challenge Hypothesis” has been proposed to explain socially-driven changes in circulating androgen levels, the same prediction may apply to transient changes in whole-body measures. However, when using whole-body androgen levels, it should be kept in mind that the levels measured do not only reflect circulating hormones but also those present in steroidogenic tissues such as the gonads, the inter-renal gland or the brain (Oliveira and Gonçalves, 2008), and that not all gonadal and/or brain androgens are released in circulation, such that a fraction of them may have local rather than systemic action. This could make the use of whole-body androgen measures problematic for testing predictions of the “Challenge Hypothesis”. However, at least in the case of gonadal androgens, which most probably are the major source of the whole-body measures, a correlation is known to exist between gonadal and circulating plasma levels in other teleost species (e.g. peacock blenny, *Salaria pavo*, Oliveira et al., 2001). Thus, it is reasonable to consider whole-body androgen levels as a proxy for circulating levels in small sized species. It is also important to note here that other indirect androgen measures have also been widely used to test predictions of the “Challenge Hypothesis” and/or to characterize socially driven changes in androgens in general (e.g. fish holding-water: Hirschenhauser et al., 2004; urine in fish and primates: Aires et al., 2015; Muller and Wrangham, 2004; Oliveira et al., 1996; or feces in birds and primates: Cristóbal-Azkarate et al., 2006; Hirschenhauser et al., 2000; Muehlenbein et al., 2004). Finally, it should also be mentioned that despite the fact that our group has recently developed a non-invasive method to assay steroids from zebrafish holding-water (Félix et al., 2013), we decided not to use it here because although it is reliable for cortisol its effectiveness for measuring sex steroids is compromised by the presence of binding globulins in the gills that have a high affinity for them (Miguel-Queralt et al., 2004; Miguel-Queralt and Hammond, 2008).

In summary, this study confirms the occurrence of a high magnitude [as indicated by the high effect sizes, i.e. >0.8 (Cohen, 1988) of the reported significant results] androgen response to an acute social challenge in a shoaling species, which supports an extension of the predictions of the “Challenge Hypothesis” to gregarious species.

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References

Abril-de-Abreu, R., Cruz, A.S., Oliveira, R.F., 2015. Social dominance modulates eavesdropping in zebrafish. R. Soc. Open Sci. 2, 150220.

Aires, R.F., Oliveira, G.A., Oliveira, T.F., Ros, A.F.H., Oliveira, R.F., 2015. Dear enemies elicit lower androgen responses to territorial challenges than unfamiliar intruders in a cichlid fish. PLoS One 10, e0137705.

Archer, J., 2006. Testosterone and human aggression: an evaluation of the challenge hypothesis. Neurosci. Biobehav. Rev. 30, 319–345.

Arnott, G., Ashton, C., Elwood, R.W., 2011. Lateralization of lateral displays in convict cichlids. Biol. Lett. 7, 683–685.

Barcellos, L.J.G., Ritter, F., Kreutz, L.C., Quevedo, R.M., da Silva, L.B., Bedin, A.C., Finco, J., Cericato, L., 2007. Whole-body cortisol increases after direct and visual contact with a predator in zebrafish, *Danio rerio*. Aquaculture 272, 774–778.

Borg, B., 1994. Androgens in teleost fishes. Comp. Biochem. Physiol. C 109, 219–245.

Cachat, J., Stewart, A., Grossman, L., Gaikwad, S., Kadri, F., Chung, K.M., Wu, N., Wong, K., Roy, S., Suciu, C., Goodspeed, J., Elegante, M., Bartels, B., Elkhayat, S., Tien, D., Tan, J., Denmark, A., Gilder, T., Kyzar, E., Dileo, J., Frank, K., Chang, K., Utterback, E., Hart, P., Kalueff, A.V., 2010. Measuring behavioral and endocrine responses to novelty stress in adult zebrafish. Nat. Protoc. 5, 1786–1799.

Cohen, J., 1988. Statistical Power Analysis for the Behavioral Sciences. Second ed. Hillsdale, New Jersey.

Cristóbal-Azkarate, J., Chavira, R., Boeck, L., Rodríguez-Luna, E., Veàl, J.J., 2006. Testosterone levels of free-ranging resident mantled howler monkey males in relation to the number and density of solitary males: a test of the challenge hypothesis. Horm. Behav. 49, 261–267.

Cruz, A.S., Oliveira, R.F., 2015. Audience effects and aggressive priming in agonistic behaviour of male zebrafish, *Danio rerio*. Anim. Behav. 107, 269–276.

Desjardins, J.K., Fernald, R.D., 2010. What do fish make of mirror images? Biol. Lett. 6, 744–747.

Dijkstra, P.D., Schaafsma, S.M., Hofmann, H.A., Groothuis, T.G., 2012. 'Winner effect' without winning: unresolved social conflicts increase the probability of winning a subsequent contest in a cichlid fish. Physiol. Behav. 105, 489–492.

Dzieweczynski, T.L., Eklund, A.C., Rowland, W.J., 2006. Male 11-ketotestosterone levels change as a result of being watched in Siamese fighting fish, *Betta splendens*. Gen. Comp. Endocrinol. 147, 184–189.

Elwood, R.W., Stoilova, V., Mcdonnell, A., Earley, R.L., Arnott, G., 2014. Do mirrors reflect reality in agonistic encounters ? A test of mutual cooperation in displays. Anim. Behav. 97, 63–67.

Engeszer, R.E., Ryan, M.J., Parichy, D.M., 2004. Learned social preference in zebrafish. Curr. Biol. 14, 881–884.

Félix, A.S., Faustino, A.I., Cabral, E.M., Oliveira, R.F., 2013. Non-invasive measurement of steroid hormones in zebrafish holding-water. Zebrafish 10, 1–6.

Filby, A.L., Paull, G.C., Bartlett, E.J., Van Look, K.J.W., Tyler, C.R., 2010. Physiological and health consequences of social status in zebrafish (*Danio rerio*). Physiol. Behav. 101, 576–587.

Fuzzen, M.L., Van Der Kraak, G., Bernier, N.J., 2010. Stirring up new ideas about the regulation of the hypothalamic–pituitary–interrenal axis in zebrafish (*Danio rerio*). Zebrafish 7, 349–358.

Galhardo, L., Oliveira, R.F., 2014. The effects of social isolation on steroid hormone levels are modulated by previous social status and context in a cichlid fish. Horm. Behav. 65, 1–5.

Gonçalves, D.M., Oliveira, R.F., 2010. Hormones and sexual behavior of teleost fishes. In: Norris, D.O. (Ed.), Hormones and Reproduction in Vertebrates, Volume 1 — Fishes. Elsevier, New York, pp. 119–147.

Goymann, W., Landys, M.M., Wingfield, J.C., 2007. Distinguishing seasonal androgen responses from male–male androgen responsiveness—revisiting the challenge hypothesis. Horm. Behav. 51, 463–476.

Hannes, R.P., 1984. Androgen and corticoid levels in blood and body extracts of high- and low-ranking swordtail males (*Xiphophorus helleri*) before and after social isolation. Z. Tierpsychol. 66, 70–76.

Hirschenhauser, K., Oliveira, R.F., 2006. Social modulation of androgens in male vertebrates: meta-analyses of the challenge hypothesis. Anim. Behav. 71, 265–277.

Hirschenhauser, K., Möstl, E., Wallner, B., Dittami, J., Kotrschal, K., 2000. Endocrine and behavioural responses of male greylag geese (*Anser anser*) to pairbond challenges during the reproductive season. Ethology 106, 63–77.

Hirschenhauser, K., Taborsky, M., Oliveira, T., Canàrio, A.V.M., Oliveira, R.F., 2004. A test of the “challenge hypothesis” in cichlid fish: simulated partner and territory intruder experiments. Anim. Behav. 68, 741–750.

Hirschenhauser, K., Winkler, H., Oliveira, R.F., 2003. Comparative analysis of male androgen responsiveness to social environment in birds: the effects of mating system and paternal incubation. Horm. Behav. 43, 508–519.

Hirschenhauser, K., Wittek, M., Johnston, P., Möstl, E., 2008. Social context rather than behavioral output or winning modulates post-conflict testosterone responses in Japanese quail (*Coturnix japonica*). Physiol. Behav. 95, 457–463.

Lachlan, R.F., Crooks, L., Laland, K.N., 1998. Who follows whom? Shoaling preferences and social learning of foraging information in guppies. Anim. Behav. 56, 181–190.

Leitz, T., 1987. Social control of testicular steroidogenic capacities in the Siamese fighting fish *Betta splendens* Regan. J. Exp. Zool. 244, 473–478.

Miguel-Queralt, S., Hammond, G.L., 2008. Sex hormone-binding globulin in fish gills is a portal for sex steroids breached by xenobiotics. Endocrinology 149, 4269–4275.

Miguel-Queralt, S., Knowlton, M., Avvakumov, G.V., Al-Nouno, R., Kelly, G.M., Hammond, G.L., 2004. Molecular and functional characterization of sex hormone binding globulin in zebrafish. Endocrinology 145, 5221–5230.

Muehlenbein, M.P., Watts, D.P., Whitten, P.L., 2004. Dominance rank and fecal testosterone levels in adult male chimpanzees (*Pan troglodytes schweinfurthii*) at Ngogo, Kibale National Park, Uganda. Am. J. Primatol. 64, 71–82.

Muller, M.N., Wrangham, R.W., 2004. Dominance, aggression and testosterone in wild chimpanzees: a test of the ‘challenge hypothesis’. Anim. Behav. 67, 113–123.

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., Canário, A.V.M., 2011. Nemo through the looking-glass: a commentary on Desjardins & Fernald. Biol. Lett. 7, 487–488.

Oliveira, R.F., Gonçalves, D.M., 2008. Hormones and social behaviour of teleost fish. In: Magnhagen, C., Braithwaite, V.A., Forsgren, E., Kapoor, B.G. (Eds.), Fish Behaviour. Science Publishers Inc., Enfield, N.H., pp. 61–150.

Oliveira, R.F., Almada, V.C., Canário, A.V.M., 1996. Social modulation of sex steroid concentrations in the urine of male cichlid fish *Oreochromis mossambicus* (Teleostei: Cichlidae). Horm. Behav. 30, 2–12.

Oliveira, R.F., Canário, A.V.M., Almada, V.C., Gonçalves, E.J., Forsgren, E., 2001. Androgen levels and social interactions in breeding males of the peacock blenny, *Salaria pavo*. J. Fish Biol. 58, 897–908.

Oliveira, R.F., Carneiro, L.A., Canário, A.V.M., 2005. No hormonal response in tied fights. Nature 437, 207–208.

Oliveira, R.F., Hirschenhauser, K., Carneiro, L.A., Canario, A.V.M., 2002. Social modulation of androgen levels in male teleost fish. Comp. Biochem. Physiol. B 132, 203–215.

Oliveira, R.F., Silva, J.F., Simões, J.M., 2011. Fighting zebrafish: characterization of aggressive behavior and winner–loser effects. Zebrafish 8, 73–81.

Paull, G.C., Filby, A.L., Giddins, H.G., Coe, T.S., Hamilton, P.B., Tyler, C.R., 2010. Dominance hierarchies in zebrafish (*Danio rerio*) and their relationship with reproductive success. Zebrafish 7, 109–117.

Pavlidis, M., Sundvik, M., Chen, Y.-C., Panula, P., 2011. Adaptive changes in zebrafish brain in dominant–subordinate behavioral context. Behav. Brain Res. 225, 529–537.

Pottinger, T.G., Calder, G.M., 1995. Physiological stress in fish during toxicological procedures: a potentially confounding factor. Environ. Toxicol. Water Qual. 10, 135–146.

Ramsay, J.M., Feist, G.W., Varga, Z.M., Westerfield, M., Kent, M.L., Schreck, C.B., 2006. Whole-body cortisol is an indicator of crowding stress in adult zebrafish, *Danio rerio*. Aquaculture 258, 565–574.

Ramsay, J.M., Feist, G.W., Varga, Z.M., Westerfield, M., Kent, M.L., Schreck, C.B., 2009. Whole-body cortisol response of zebrafish to acute net handling stress. Aquaculture 297, 157–162.

Spence, R., Smith, C., 2005. Male territoriality mediates density and sex ratio effects on oviposition in the zebrafish, *Danio rerio*. Anim. Behav. 69, 1317–1323.

Teles, M.C., Dahlbom, S.J., Winberg, S., Oliveira, R.F., 2013. Social modulation of brain monoamine levels in zebrafish. Behav. Brain Res. 253, 17–24.

Vital, C., Martins, E.P., 2013. Socially-central zebrafish influence group behavior more than those on the social periphery. PLoS One 8, e55503.

Wingfield, J.C., Hegner, R.E., Dufty, A.M., Ball, G.F., 1990. The “challenge hypothesis”: theoretical implications for patterns of testosterone secretion, mating systems, and breeding strategies. Am. Nat. 136, 829–846.