Rising to the challenge? Inter-individual variation of the androgen response to social interactions in cichlid fish

Ana S. Félix[a,b], António Roleira[a], Rui F. Oliveira[a,b,c,*]

[a] ISPA - Instituto Universitário, Rua Jardim do Tabaco 34, 1149-041 Lisboa, Portugal
[b] Instituto Gulbenkian de Ciência, Rua da Quinta Grande 6, 2780-156 Oeiras, Portugal
[c] Champalimaud Neuroscience Programme, Champalimaud Centre for the Unknown, Avenida Brasília, 1400-038 Lisboa, Portugal

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ABSTRACT

The Challenge Hypothesis (Wingfield et al. Am. Nat. 136, 829-846) aims to explain the complex relationship between androgens and social interactions. Despite its well acceptance in the behavioral endocrinology literature, several studies have failed to found an androgen response to staged social interactions. Possible reasons for these inconsistencies are the use of single sampling points that may miss the response peak, and the occurrence of inter-individual variability in the androgen response to social interactions. In this study we addressed these two possible confounding factors by characterizing the temporal pattern of the androgen response to social interactions in the African cichlid, *Oreochromis mossambicus,* and relating it to inter-individual variation in terms of the individual scope for androgen response (i.e. the difference between baseline and maximum physiological levels for each fish) and behavioral types. We found that the androgen response to territorial intrusions varies between individuals and is related to their scope for response. Individuals that have a lower scope for androgen response did not increase androgens after a territorial intrusion but were more aggressive and exploratory. In contrast males with a higher scope for response had fewer aggressive and exploratory behaviors and exhibited two peaks of KT, an early response 2–15 min after the interaction and a late response at 60–90 min post-interaction. Given that the pharmacological challenge of the Hypothalamic-Pituitary-Gonad axis only elicits the late response, we suggest that these two peaks may be regulated by different physiological mechanisms, with the early response being mediated by direct brain-gonad neural pathways. In summary, we suggest that determining the temporal pattern of the androgen response to social interactions and considering inter-individual variation may be the key to understanding the contradictory results of the Challenge Hypothesis.

1. Introduction

Almost 30 years ago, Wingfield et al. (1990) introduced the Challenge Hypothesis as a framework to study the androgen response to social interactions. Since then, their essay has been a landmark for behavioral endocrinologists that aim to understand the complex relationship between androgens and the social environment. According to this model, constitutive androgen circulating levels (constitutive baseline, *a*) occur during the non-breeding phase, while at the onset of the breeding season they increase up to the concentration needed for the full development of the gonads, the development of secondary sex characteristics and for the expression of reproductive behaviors (breeding baseline, *b*) (Wingfield et al., 1990). Then, androgens can further rise above the breeding baseline and reach a physiological maximum (*c*) in response to social interactions, either with males or

with sexually receptive females (Wingfield et al., 1990). Thus, the social interactions of an individual will determine its androgen levels. Moreover, the Challenge Hypothesis generates a number of predictions regarding the seasonal patterns of androgen social responsiveness [quantified by the ratio (*c-a*)/(*b-a*)] in seasonal breeders according to the mating system and parental care type of the species. For example, since androgens interfere with paternal care, males from species that provide parental care should have low androgen levels during parental phase that rise in response to male or female interactions (high androgen responsiveness), whereas species in which males invest less in parental care are expected to have higher androgen levels but lower androgen responsiveness to social interactions (Wingfield et al., 1990).

Although the Challenge Hypothesis was initially proposed based on comparative data from bird species, it has been extensively tested across all vertebrate taxa, including teleost fish (Hirschenhauser and

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Oliveira, 2006; Hirschenhauser et al., 2004; Oliveira, 2004). Overall, the predictions regarding the seasonal variations in androgen levels have been confirmed (Goymann et al., 2007), but many of the published studies, even in birds, failed to observe the predicted androgen response to simulated social challenges (e.g., rodents: Fuxjager et al., 2010; dwarf mongooses: Creel et al., 1993; amphibians: de Assis et al., 2012; fish: Ros et al., 2014; reptiles: Baird et al., 2014; birds: Moore et al., 2004). Recently, it has been argued that one possible explanation for these contradictions is the fact that most studies so far have focused on androgen responses to male-male interactions (i.e. territorial intrusions or staged fights), and that male-female interactions would contribute more to the observed seasonal patterns (Goymann et al., 2019). However, there are other possible explanations for the failure in detecting the androgen response to social interactions, namely using inappropriate sampling points that miss the peak of response due to lack of knowledge on the time courses of the response for each studied species and the occurrence of inter-individual variability in androgen response associated to behavioral variation (e.g. personality types).

Indeed, the temporal dynamics of the androgen response to social interactions has only been studied in few species and there is significant variation in the observed patterns. For instance, the response of testosterone in males to the presence of a receptive females peaks between 30 min and 60 min after exposure in male Sprague-Dawley rats (Kamel and Frankel, 1978), whereas in male mice (CBA strain) it peaks at 20 min in Winter and at 40 min in Summer (Amstislavskaya and Popova, 2004). Similarly, aggressive encounters elicit a testosterone peak in winners 45 min after a fight in California mice (*Peromyscus californicus*) (Marler et al., 2005) and at 60 min post-fight in swordtail fish (*Xiphophorus helleri*) (Hannes et al., 1984). Thus, without knowing the temporal dynamics of the androgen response to social interactions for their studied species and using reported sampling points for other species, some studies with negative results may have simply missed the androgen peak they were aiming to characterize, highlighting the need for the characterization of the time course of the response for each species.

On the other hand, few studies account for inter-individual variation in hormonal responses. Usually, comparisons of androgen responsiveness are made between species averaging all sampled individuals (e.g., Goymann, 2009; Hirschenhauser et al., 2003, 2004; Oliveira et al., 2002; Wingfield et al., 1990), ignoring the possible occurrence of alternative phenotypes that may cancel each other in the sample. By 1987, (Bennett, 1987) already emphasized the need to focus on biological differences among individuals and to shift our attention from the 'tyranny of the Golden Mean', particularly in physiological studies. Indeed, although the function and mechanisms that underpin individual variation are not fully understood, several authors have highlighted the importance of this issue in the context of endocrinology (Hau and Goymann, 2015; Kempenaers et al., 2008; Williams, 2008). Actually, for the same population, variation in hormone levels among individuals is quite impressive (e.g. up to two orders of magnitude, (Kempenaers et al., 2008; see also Williams, 2008) and may be related to fitness (Hau and Goymann, 2015). Such differences between individuals in terms of baseline and/or maximum levels could influence the scope for androgen responsiveness leading to inter-individual differences within the same species.

Moreover, inter-individual variation of the androgen response can be related to intrinsic psychological features, such as observed in the stress response (Koolhaas et al., 1999). In recent years, a considerable amount of literature has been published on inter-individual variation in behavior profiles. Contrasting patterns are observed whenever individuals behave and interact with their environment. Several definitions with somewhat similar meanings have been proposed for consistent differences between individuals. The term 'temperament' (or personality) is generally understood as the consistency of behavioral differences between individuals over time and across situations (Caramachi et al., 2013; Réale et al., 2007). Distinct behavioral traits

(or axes) have been proposed: shyness-boldness (in risky situations, e.g. predator), exploration-avoidance (in new situations), activity (in non-risky and non-novel situations), aggressiveness (towards conspecifics) and sociability (Réale et al., 2007; Sih et al., 2004). It is worth noting that, in a population, the distribution of individuals along these axes is expected to follow a continuum, rather than a bimodal distribution (Réale et al., 2007). If a set of behavioral traits correlate between each other, one may define it as a 'behavioral syndrome' (Bell, 2007; Sih et al., 2004), which could mean that the traits are regulated by a common neuroendocrine, genetic or neurobiological mechanism (Coppens et al., 2010; Sih et al., 2004). For instance, the best known behavioral syndrome is the proactive-reactive syndrome, studied in the context of stress research to distinguish animals with opposing stress-coping styles (see, for example, Coppens et al., 2010; Koolhaas et al., 1999; Øverli et al., 2007; Young et al., 2011). Proactive individuals are simultaneously bolder, more aggressive and active in response to challenges, show higher exploration rates and, in general, a low Hypothalamus-Pituitary-Adrenals (HPA) axis activity and high sympathetic reactivity. In contrast, reactive individuals seem consistently shy, less aggressive and active, usually freeze in stressful situations and have higher HPA axis and lower sympathetic responses (Koolhaas et al., 1999).

The aim of our study is to characterize the temporal pattern of the androgen response to social (male-male) interactions, taking into account the scope for response of each individual and to relate it to inter-individual variation in behavior profiles. For this purpose, we studied Mozambique tilapia, *Oreochromis mossambicus*, a freshwater fish with a lek-mating system where breeding (dominant) males, which do not show parental care (Fryer and Iles, 1972), aggregate densely in mating territories, where they dig and defend spawning pits (Oliveira and Almada, 1998). A first longitudinal study of weekly territorial challenges followed by androgen sampling at different post-challenge time points (Social challenge experiment), was conducted to characterize the time course curve for each animal. A second longitudinal study (Physiological challenge experiment) was conducted to characterize the time course of the androgen response to a physiological challenge (GnRH injection), Our goal was to compare temporal circulating androgen levels obtained in response to social interactions and those elicited by Hypothalamus-Pituitary-Gonads (HPG) axis stimulation to assess the involvement of the HPG in the observed androgen response to social interactions.

2. Materials and methods

2.1. Animals and housing

O. mossambicus adult fish from a stock held at ISPA were used in this experiment. Fish were maintained in glass tanks (120 × 40 × 50 cm, 240 l) with a fine gravel substrate. Each tank was supplied with a double filtering system (gravel and external biofilter) and continuous aeration. Water quality was analized twice per month for nitrites (0.2–0.5 ppm), ammonia (< 0.5 ppm, Pallintest kit) and pH (6.0–6.2). Fish were kept at a temperature of 26 ± 2 °C, a 12 L:12D photoperiod, and fed with commercial cichlid floating sticks. *O. mossambicus* males present two distinct phenotypes: dominants are usually larger, dark colored, establish territories and attract females; while subordinates have a silver color pattern similar to females and fail to establish territories (Oliveira and Almada, 1998). Thirty-six focal dominant males (2.5–3 years old) were used for the experiments described below. Males' social status was monitored several times per week and territorial males were identified by nuptial black coloration and exhibition for at least 1 week of reproductive behavior, including territorial defense and digging of a spawning pit in the substrate (Oliveira and Almada, 1996).
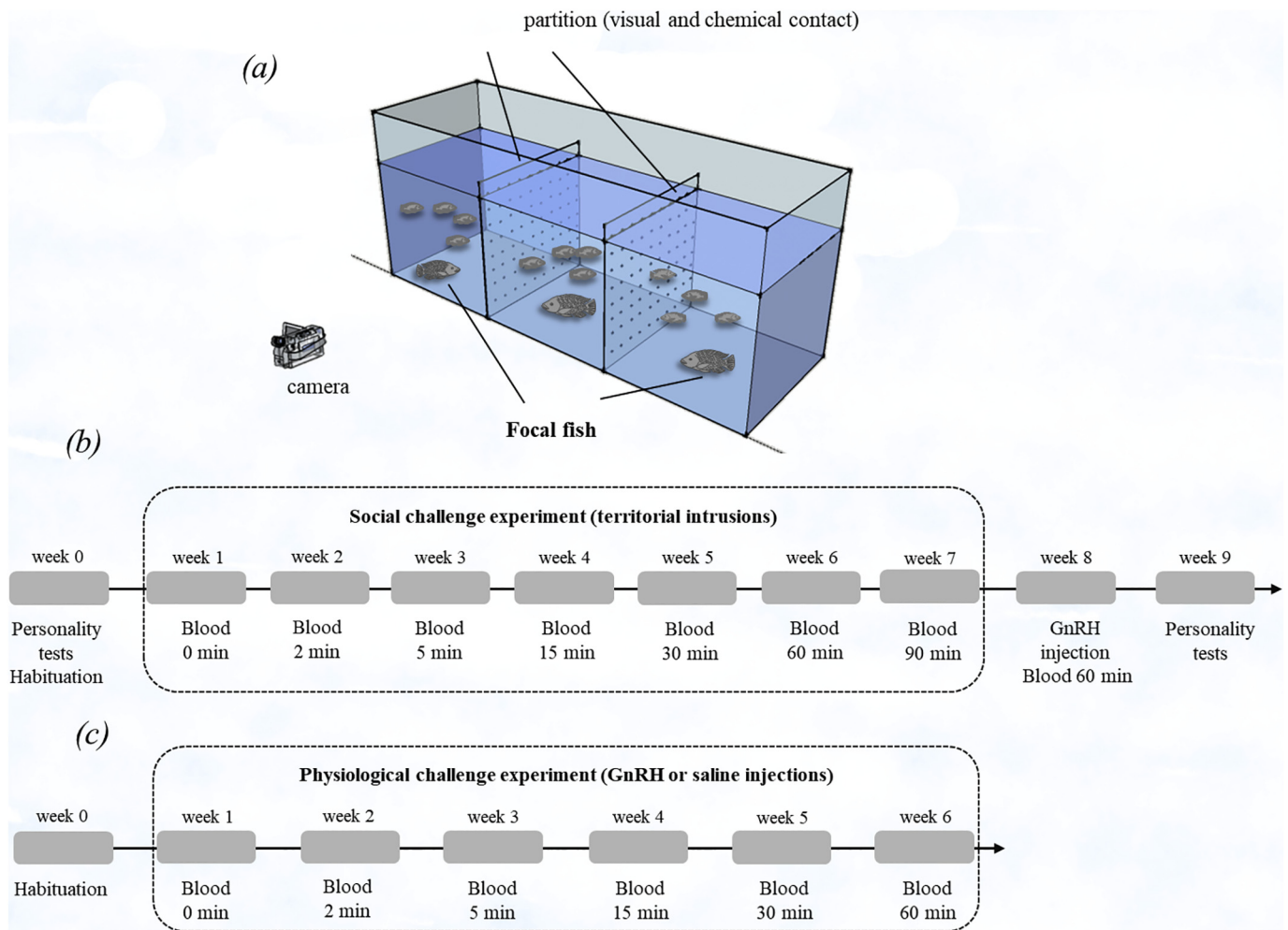
Fig. 1. Experimental design. (*a*) 3D diagram of the experimental setup. Experimental tanks were divided in compartments by partitions which allowed chemical and visual contact between them. Each compartment contained a dominant male and 4 females. Each side compartment held a focal male. (*b*) Timeline of the Social challenge experiment (within-subject design). In the first week of experiment, focal males were phenotyped for behavioral profiles (personality tests). In the following weeks, focal males were exposed to territorial intrusions and their blood sampled at 2, 5, 15, 30, 60 and 90 min after the intrusion. In week 8, focal males were injected with sGnRH and sampled after 60 min to measure the androgen's physiological maximum of each fish. In the final week of experiment, focal males were phenotyped for behavioral profiles (personality tests). (*c*) Timeline of Physiological challenge experiment (within-subject design). Focal males were i.p. injected either with sGnRH (GnRH treatment group) or with a saline solution (control group) once a week and blood was sampled 2, 5, 15, 30 or 60 min after the injection.

2.2. Social challenge experiment

2.2.1. Experimental setup

Twenty focal dominant males (mean body mass ± SEM: 48.03 g ± 1.99 g; mean standard length ± SEM: 11.23 cm ± 0.16 cm) were phenotyped for their behavioral profile (see below: personality tests), lightly anaesthetized (MS-222, Pharmaq; 300 ppm) to be weighed and measured, and then introduced into experimental tanks (Fig. 1). Each experimental tank (40 × 50 × 120 cm) was divided into 3 compartments with transparent and holed partitions so that chemical and visual contact was possible between compartments. Each compartment contained a dominant male and 4 females. Each side compartment held a focal male. Hence, our experimental setup allowed focal males to interact with other males and females in a simulated semi-natural environment. After 1 week of habituation, focal males were allowed to interact with a male (see below) introduced to their compartment. The intruder male was removed 3 min after the first aggressive behavior of the focal male towards the intruder. The experiment was run for several weeks to obtain several sampling time points after intrusion. Focal males were randomly removed from the tank at 2, 5, 15, 30, 60 or 90 min after intrusion to collect blood and returned to the experimental tank. A sampling time point of 0 min

corresponds to a week where there was no intrusion. Intruder males were isolated individually also in experimental tanks with 4 females per compartment; they were also territorial males but selected from different stock tanks from those of focal males. Focal males were always larger than intruders (mean body mass ± SEM: 25.31 g ± 0.96 g; mean standard length ± SEM: 9.07 cm ± 0.14 cm) to ensure the focal male's social advantage. Focal males were subjected to a different intruder each week to control for possible familiarity cues and promote more aggressiveness from focal fish towards stranger intruders (Aires et al., 2015). Interactions between focal and intruder males were video recorded for subsequent behavioral analysis. In the next-to-last week of experiment, focal males were injected with sGnRH (1000 µg/kg; sGnRH analog, (Des-Gly[10],D-Ala[6],Pro-NHEt[9])-LHRH (salmon); Bachem #H-7525) and sampled after 60 min to measure the maximum physiological level of each male's androgens. This procedure followed the proposed use of GnRH challenges to estimate maximum physiological levels in the scope of testing the challenge hypothesis (e.g., Apfelbeck and Goymann, 2011). In the final week of experiment, focal males were phenotyped for behavioral profiles (personality tests) using the same behavioral protocol that was used before the start of the experiment to ascertain trait consistency over time, a main requisite of personality. To reduce behavioral or hormonal fluctuations associated with natural
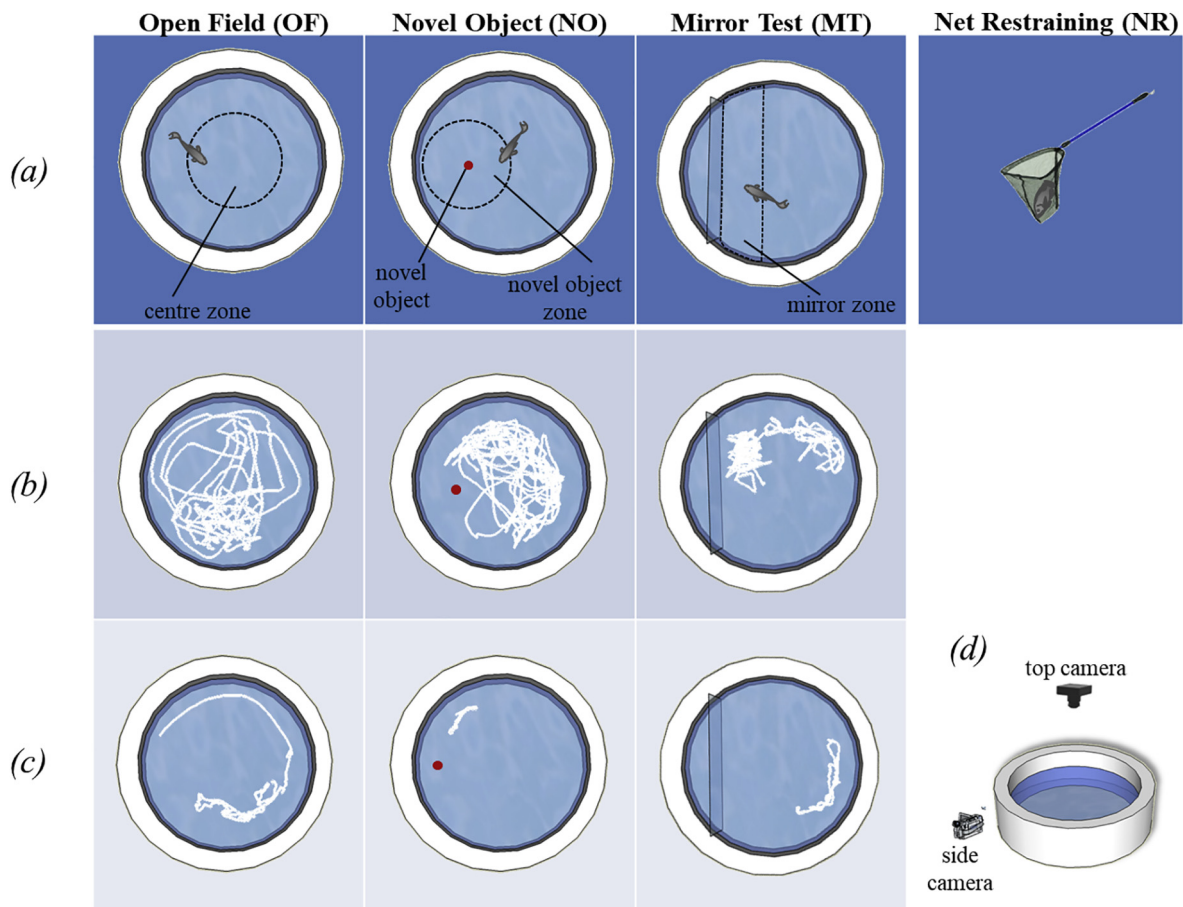
Fig. 2. Personality tests. *(a)* Behavioral testing procedures: open field (OF), novel object (NO), mirror test (MT) and net restraining (NR). *(b)* and *(c)* representative examples of video-tracking of: *(b)* individual with high level of exploration in OF and NO and that fought with the mirror; and *(c)* individual with low level of exploration in OF and NO and that did not fight with the mirror. *(d)* 3D diagram of the experimental arena.

circadian rhythm, personality tests were always conducted in the afternoon, while the territorial intrusions were always conducted in the morning.

sGnRH dose was selected based on a dose-response pilot experiment where four different doses and a saline control were tested in male fish. We selected the dose that produced the highest significant increase in circulating androgens above baseline levels (Supplementary Fig. S1).

2.2.2. Personality tests

To determine if the androgen response to social interaction is related to behavioral types, we tested individuals on two personality dimensions: exploration-avoidance and aggressiveness (Réale et al., 2007). The focal male was subjected twice (in the first and the last week of the main experiment, see above) to a battery of behavioral tests to assess individual variability and trait consistency. In each session, the focal male was exposed to 4 behavioral tests: (1) open field, (2) novel object, (3) mirror test and (4) net restraining (Fig. 2). Tests were performed consecutively in the same order, but the order of males used in each session was random. The experimental arena consisted on an unfamiliar circular white tank (external diameter: 54 cm; filled to a depth of 12 cm; 20 l of water) with a webcam (Logitech webcam C170) placed overhead for a top-down view. A video camera (Sony DCR-SR58E) was placed on the side to enable better discrimination of aggressive behaviors in the mirror test. To minimize disturbance from the surroundings, opaque divisions were placed around the arena. The experimental arena was cleaned at the end of each individual session and the water was replaced with clean matured water.

Open Field Test (OF) – The male was carefully placed in the arena.

After 30 s of acclimation, the test phase was initiated and the male was tracked using commercial video tracking software (EthoVision® XT 8.0, Noldus Inc. the Netherlands). Two zones were defined for assessing thigmotaxis (a wall-seeking spatial strategy associated with anxiety, (Champagne et al., 2010) and exploratory behavior in a novel but limited environment: a 10 cm outer zone (near the arena walls) and the remaining inner area as the centre zone. The following behaviors were recorded for 10 min: total time in movement, total distance moved, total time spent in the centre zone of the arena, latency to enter the centre zone, number of times spent in the centre zone of the arena, and distance moved in the centre zone. Males that never entered the centre zone were given a maximum latency of 600 s.

Novel Object Test (NO) – Immediately after the OF assay, a weighted red object was introduced in the arena using a fishing line. To keep the object novel, males were presented with a red Lego brick (3 × 3 × 3 cm) in one trial and a red ball of similar dimensions (4 cm diameter) in the other trial. This test was conducted to estimate neophobia/neophilia behavior, a particular dimension of exploration-avoidance temperament trait (Réale et al., 2007). Two zones were defined for assessing behavioral measures: the novel object zone was a circular 10 cm radius zone around the object; the remaining area. The following behaviors were recorded for 10 min: total time in movement, total distance moved, total time spent in the novel object zone, latency to enter the novel object zone, number of times spent in the novel object zone and distance moved in the novel object zone. Males that never entered the novel object zone were given a maximum latency of 600 s.

Mirror Test (MT) – At the end of the NO, the novel object was gently removed using the fishing line. The male was given 2 min to settle, then

a mirror (dimension: 21 × 30 cm) was placed in the arena leant in a way that prevented males from going behind it during the trial. Since fish do not recognize themselves in mirror, they fight their own image as if it is a conspecific intruder (Oliveira et al., 2005). This test was conducted to assess the male's agonistic reaction in a standardized assay. Two zones were defined to obtain behavioral measures: the mirror zone was a 10 cm-width area near the mirror; the remaining area. The following behaviors were recorded for 5 min: total time in movement, total distance moved, total time spent in the mirror zone, latency to enter the mirror zone, number of times spent in the mirror zone and distance moved in the mirror zone. Additionally, the number and duration of aggressive behaviors (lateral displays, frontal displays, bites, tail beating) and latency to attack were analyzed using Observer XT software (Noldus technology, version 5, Netherlands). Males that never entered the mirror zone were given a maximum latency of 300 s.

Net Restraining Test (NR) – At the end of the MT, the male was held in a net, out of water, for 1 min. The following behaviors were recorded: number of escape attempts and the total time spent in escape attempts. This is an assay that has been used to evaluate escape behavior in other fish species (e.g., gilthead sea bream, *Sparus aurata L.*, in Arends et al., 1999; Castanheira et al., 2013; flatfish Senegalese sole, *Solea senegalense*, in Silva et al., 2010; Nile tilapia, *Oreochromis niloticus*, in Martins et al., 2011).

2.3. Physiological challenge experiment

This study was conducted to characterize the time course of the androgen response to a GnRH challenge. On the first day of experiment, focal males were lightly anaesthetized (MS-222, Pharmaq; 300 ppm) to be weighed and measured, and then introduced into the same experimental tanks used in the Social Challenge Experiment (Fig. 1). Sixteen focal dominant males (mean body mass ± SEM: 51.65 g ± 2.93 g; mean standard length ± SEM: 11.80 cm ± 0.22 cm) were used. Focal males were arbitrarily assigned to the GnRH treatment group (n = 8) or to the control group (n = 8). All males had intraperitoneal injections once a week, treatment group (G group) with a GnRH analog (100 μg/kg), controls (V group) with a saline vehicle solution (0.9% sodium chloride). The experiment was run for several weeks to obtain samples at several time points after the injection. Focal males were randomly removed after 2, 5, 15, 30 or 60 min after injection to collect blood and returned to the experimental tanks. The sampling time point of 0 min corresponds to a week where there was no injection.

2.4. Behavioral observations

Behavior of focal males during territorial intrusions (main experiment) was analyzed using Observer XT software (Noldus technology, version 5, Netherlands), based on the ethogram provided by Baerends and Baerends-van Roon (1950). Relevant behavioral patterns were identified to measure male aggressiveness towards the intruder, the neighbor or the females (i.e. bites, displays, buttings, chasing, tail beating). Other behaviors (nipping, swimming, floating, courting, glass interactions) were also quantified. We recorded the frequency and latency of the reported behaviors, as well as the attack latency (i.e., time between the beginning of the recording period and the first aggressive behavior). Similarly, for personality tests the aggressive behavior of focal males during the Mirror Test (MT) and the time spent performing escape attempts in the Net Restraining Test (NR) was analyzed using the same software. Other variables measured in the personality tests were obtained with EthoVision XT 8.0 (Noldus Inc. the Netherlands).

2.5. Blood sampling

Males were anaesthetized (MS-222, Pharmaq; 450 ppm) and blood was collected from the caudal vein using heparinized 25-gauge needles. Blood sampling always took place within 4 min of the induction of

anaesthesia to prevent possible effects of handling stress on steroids levels (Foo and Lam, 1993). Blood samples were centrifuged (10 min, 3000 g, 4 °C) and plasma was stored at −20 °C until further processing.

2.6. Hormone assays

11-ketotestosterone (KT), testosterone (T) and cortisol (F) were extracted from plasma samples by adding diethyl-ether (Merck). Samples were then agitated for 20 min, centrifuged (5 min, 163 g, 4 °C) for phase separation and kept at −80 °C for 15 min to freeze the water phase and separate the ether fraction (containing the free steroid). This process was repeated twice to obtain higher extraction efficiency. The ether fraction was evaporated with a Speedvac (Savant SC1101) and the dried organic phase was re-suspended in phosphate buffer. Steroid concentrations were measured by radioimmunoassay using a testosterone antibody from Research Diagnostics Inc. (#WLI-T3003, rabbit anti-testosterone) and cortisol antibody from Fitzgerald (#20-CR-50, rabbit anti-cortisol). The antibody used for the 11-ketotestosterone assay was kindly donated by D. E. Kime and the corresponding specificity table was published in Kime and Manning (1982). Reactive markers used in radioimmunoassays for testosterone and cortisol were from Amersham Biosciences ([1,2,6,7-3H] Testosterone, #TRK402-250 μCi; [1,2,6,7-3H] Cortisol, #TRK407-250 μCi) while 11-ketotestosterone marker was produced in-house from marked cortisol (Kime and Manning, 1982). Samples collected from each male were run in the same assay. Inter-assay variabilities were 7.9% for KT, 8.0% for T and 11.9% for F. Intra-assay variation coefficients were 8.0%, 4.9%, 4.7%, 0.4% and 5.2% for KT; 11.5%, 5.5%, 5.1, 4.3% and 8.8% for T; 4.9% and 11.2% for F.

2.7. Data analysis

Normality of the data was tested by analyzing skewness and kurtosis values (Kline, 1998) and running Shapiro-Wilk tests. If necessary, variables were log transformed to meet parametric assumptions. Homocedascity was confirmed with Levene's test.

The androgen scope of each individual, defined as the androgen responsiveness score, was assessed by dividing KT baseline levels for KT physiological maximum (GnRH induced levels). Individuals that had values above the mean were defined as low androgen responders (LR), because they had a lower scope for response; while those that had an androgen responsiveness score below the mean were considered the high androgen responders (HR) because they had a greater scope of response. We used KT in this calculation since it has been reported as the main androgen in teleost fish (Borg, 1994) and found to respond to social interactions in this species (Hirschenhauser et al., 2004). For the Social challenge experiment, hormone levels (KT, T and F) were analyzed using a Linear Mixed Model (LMM) with time and type of androgen response (HR vs LR) as fixed effects and focal male as a random effect. Planned comparisons were used to compare steroid levels relative to baseline and the p-values were adjusted for multiple testing using the Benjamini and Hochberg (1995) procedure.

Plots of residuals, fitted values and estimated random effects were used to confirm assumptions of LMM.

Body condition index in the beginning and at the end of the experiment was calculated for each male by dividing its actual weight by its expected weight. Expected weight was assessed by the weight-length relationship obtained in a field study for *O. mossambicus* (Silva, 1985). t-tests were used to compare body mass, body size and body condition index for the two groups (LR and HR males).

We measured repeatability of behavioral variables, for which we obtained multiple measurements (personality tests and territorial intrusions). Repeatability, more generally referred to as the intra-class correlation (ICC), is defined as the proportion of phenotypic variation explained by differences between individuals (Dingemanse and Dochtermann, 2013; Lessells and Boag, 1987): Repeatability = V_{IND} /
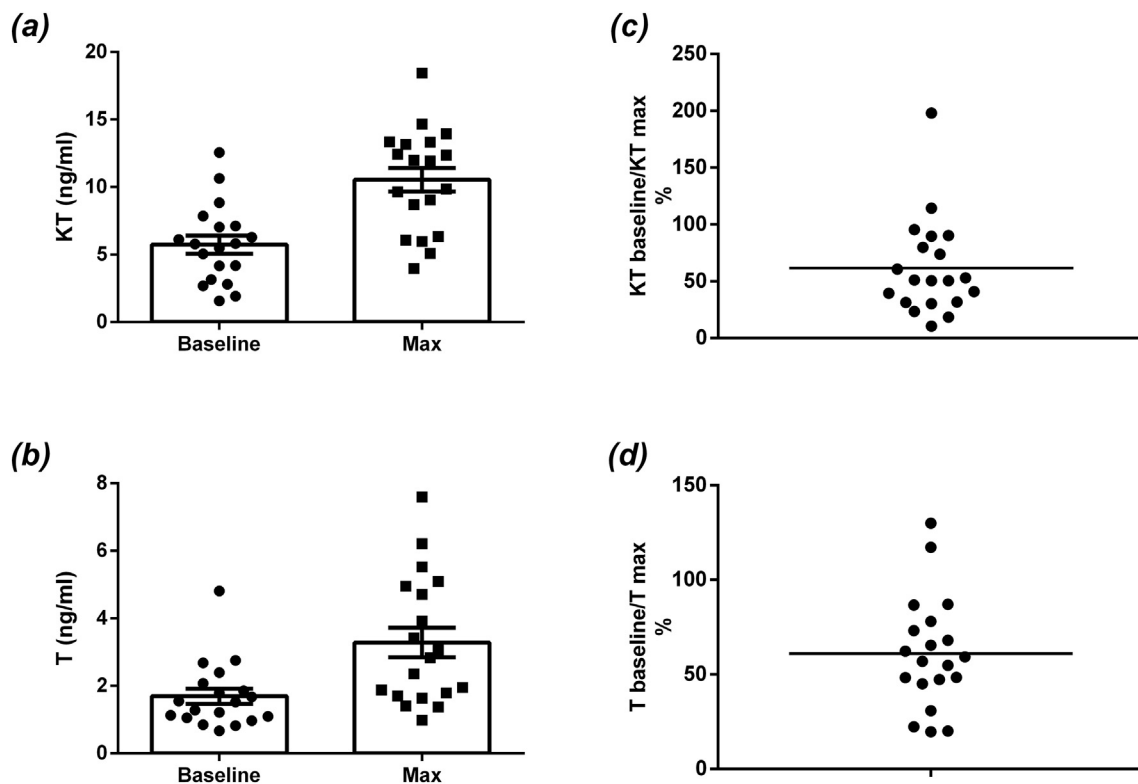
Fig. 3. Individual variation in androgens. *(a)* 11-ketotestosterone (KT); *(b)* testosterone (T); *(c)* androgen scope for KT (KT baseline/KT max); *(d)* androgen scope for T (T baseline/T max); where baseline corresponds to the week with no intrusion and max to the week of GnRH injection. Histograms in *(a)* and *(b)* show mean ± standard error of the mean (SEM), in *(c)* and *(d)* the line shows the mean. Individual values shown as symbols.

V_P, where $V_P = V_{IND} + V_e$ is the phenotypic variation, composed of the between-individual variance V_{IND} and the within-individual variance V_e. V_e represents the 'residual error' due to errors in measurements and general environmental variance. On the other hand, repeatability aims to measure the total variation that is reproducible, that is, the consistency of each trait in the population, allowing comparison across studies for the same trait and across traits in the same study (Dingemanse and Dochtermann, 2013; Harrison et al., 2018). To verify that behavioral responses reflected personality traits, we used the rtpR package (Stoffel et al., 2017) to calculate repeatability. With this package, uncertainty is measured via parametric bootstrapping and likelihood ratio tests are used for significance testing. The number of parametric bootstrap iterations for confidence interval estimation and statistical significance was set to 1000. We have not calculated repeatabilities for variables accounting for aggressive behaviors in the Mirror Test since in the second trial, none of the males fought with the mirror.

T-tests were used to compare behavioral measures that were repeatable, and assess if any of the behaviors were significantly different for the two groups (LR and HR males).

Behavioral variables of the OF were reduced with Principal Component Analysis (PCA) and different component loadings were obtained with the variable principle normalization method. Since variables were preferentially loaded on the first PCA dimension, only one component was selected which described 68.8% of the variance of behavior on the OF. This PCA component was interpreted as describing males as more or less exploratory (Supplementary Table S1). Behavioral variables of the territorial intrusion experiment were also reduced with PCA and different component loadings were obtained with the variable principle normalization method. The first PCA dimension was loaded with behavioral variables related to aggression towards intruders and females (29.7% explained variance), while the second component was related to aggression towards the neighbor (18.5% explained variance)

(Supplementary Table S2). For these PCA analyses we used the average of the behaviors between the trials. A Pearson correlation between PCA scores of the OF and PCA scores of territorial intrusions was used to examine the relationship between exploration and aggression.

For the Physiological challenge experiment, hormone levels were analyzed using a Linear Mixed Model (LMM) with time and treatment (GnRH or saline injected) as fixed effects and focal male as a random effect. Planned comparisons were used to compare steroid levels relative to baseline and the p-values were adjusted for multiple testing (Benjamini and Hochberg, 1995).

Effect sizes were computed for LMM tests (omega-squared, ω^2) and for post-hoc tests (Cohen's d).

Since we were analyzing individual variability we decided not to remove any apparent outliers or extreme values. Degrees of freedom may vary between the analyses due to missing values because of technical problems (i.e. with blood collection, RIA or video recordings).

Statistical analysis was performed using IBM SPSS® statistics v.21, STATISTICA v.10 (StatsoftInc), and R (R Core Team, 2015) with the following packages: nlme (linear mixed model), multcomp (planned comparisons), rtpR (repeatability), sjstats (effect sizes).

2.8. Ethics statement

In this study, we have kept aggressive interactions to a short period (3 min) and no signs of physical injuries were observed during any of the staged interactions. Experimental procedures used in this study were conducted in accordance with the institutional guidelines for the use of animals in experimentation and were approved both by the internal Ethics Committee of ISPA and by the Portuguese Veterinary Authority (Direção Geral de Alimentação e Veterinária, Portugal; permit number 0421/000/000/2013).

3. Results

3.1. Social challenge experiment

There was inter-individual variation in baseline levels (KT: 8.0-fold; T: 7.2-fold; F: 39.6-fold), physiological maximum (KT: 4.6-fold; T: 7.7-fold) and in the androgen scope (KT: 18.9-fold; T: 6.6-fold) of focal males (Fig. 3). Following this variation, males were grouped into low responders (LR; n = 7) or high responders (HR; n = 13) according to their androgen responsiveness score (see details in Methods). There was no significant variation in body size between LR and HR males (t (18) = 1.767, p = 0.094), but there was in body weight (beginning of experiment: t(17) = 3.089, p = 0.007; end of experiment: t (18) = 2.100, p = 0.050). Body condition index was also significantly different between LR and HR fish at the beginning (t(17) = 3.261; p = 0.005; mean (LR) ± SE = 69.91 ± 4.995; mean (HR) ± SE = 59.91 ± 6.655 values) and at the end of the experiment (t (18) = 2.099, p = 0.050; mean (LR) ± SE = 61.97 ± 7.004; mean (HR) ± SE = 55.22 ± 6.795).

The levels of KT, T and F changed significantly with time (KT: $F_{(7,117)}$ = 6.855, p < 0.0001, T: $F_{(7,116)}$ = 7.296, p < 0.0001, F: $F_{(6,98)}$ = 23.718, p < 0.0001, Table 1), but were not significantly affected by whether the fish were HR or LR (KT: $F_{1,18}$ = 0.071, p = 0.793, T: $F_{(1,18)}$ = 0.104, p = 0.751, F: $F_{(1,18)}$ = 0.395, p = 0.538; Table 1). However, the interaction between these factors was significant for KT ($F_{(7,117)}$ = 2.629, p = 0.015, Table 1). HR fish had a significant increase above baseline of KT 2 min and 5 min and then again at 90 min after the territorial intrusion (Table 1, Fig. 4a). There was no difference in KT or T in LR fish after territorial intrusions (Table 1, Fig. 4a and b). GnRH injection elicited a significant increase of both androgens only in HR fish (Table 1, Fig. 4a and b). F levels were higher than baseline between 2 min and 15 min for LR and between 2 min and 5 min for HR (Table 1, Fig. 4c).

For personality tests, only behavioral variables from the Open Field (OF) were significantly repeatable, namely the distance moved in the centre zone, total distance moved and total time in movement, with repeatability scores higher than 0.57 (Supplementary Table S3).

For territorial intrusions, most of the behavioral variables, either aggressive or non-aggressive were significantly repeatable (Supplementary Table S4). The most repeatable behavior was time spent in chases with a score of 0.587 (Supplementary Table S4).

Several repeatable behavior variables differed significantly between LR and HR males. In the OF, the total distance moved was significantly different between males (LR moved more than HR; Table 2). Other behaviors measured in the OF showed a non-significant tendency of LR to move more in the center of the arena and to spend more time in movement than HR (Table 2). During territorial intrusions, the total number of behaviors (measured as a proxy of activity), aggressive behaviors towards both the intruder and the neighbor and the aggressive behaviors towards the intruder were significantly different, with LR displaying a higher number of these behaviors than HR (Table 2). Similarly, aggressive behaviors towards the neighbor show a non-significant trend of LR to be more aggressive than HR (Table 2).

The PCA score of the OF was significantly negatively correlated with

Table 1
Effect of time and type of fish (LR vs HR) on hormone levels after territorial intrusion (Social challenge experiment). Main effects (LMM), interactions, effect sizes and planned comparisons between the baseline and the other time points.

Comparisons	Main effects			Planned comparisons					
				LR			HR		
	F	p	ω^2	z	p	d	z	p	d
KT			0.564						
Time	6.855	< .0001							
Fish type	0.071	.793							
Time × fish type	2.629	**.015**							
0 min vs 2 min				−0.869	.490	0.613	2.660	.043	0.810
0 min vs 5 min				−0.885	.490	0.392	2.579	.043	0.806
0 min vs 15 min				−1.723	.136	0.994	1.939	.123	0.734
0 min vs 30 min				−1.708	.136	1.137	0.693	.570	1.413
0 min vs 60 min				−1.862	.125	1.186	2.281	.063	0.849
0 min vs 90 min				0.264	.853	0.370	2.502	**.043**	0.942
0 min vs GnRH				0.038	.970	0.021	6.859	**< .0001**	0.982
T			0.673						
Time	7.296	< .0001							
Fish type	0.104	.751							
Time × fish type	1.413	.207							
0 min vs 2 min				−0.902	.734	0.364	0.670	.782	0.227
0 min vs 5 min				−0.701	.782	0.248	1.369	.734	0.415
0 min vs 15 min				−0.022	.996	0.007	0.946	.734	0.278
0 min vs 30 min				−0.548	.817	0.193	0.285	.988	0.053
0 min vs 60 min				−0.005	.996	0.002	1.144	.734	0.381
0 min vs 90 min				−0.115	.996	1.010	2.153	.219	0.745
0 min vs GnRH				1.106	.734	0.377	6.258	**< .0001**	1.564
F			0.698						
Time	23.718	< .0001							
Fish type	0.395	.538							
Time × fish type	1.160	.334							
0 min vs 2 min				3.533	**.002**	1.029	7.547	**< .0001**	1.750
0 min vs 5 min				3.118	**.005**	1.430	3.199	**.005**	0.956
0 min vs 15 min				2.330	**.048**	0.796	2.116	.069	1.246
0 min vs 30 min				0.319	.750	0.036	1.002	.422	1.102
0 min vs 60 min				−0.922	.428	0.467	−0.562	.627	0.098
0 min vs 90 min				−1.451	.220	1.802	−1.787	.127	0.209

11-ketotestosterone (KT); testosterone (T); cortisol (F); LR – Low responder fish; HR – High responder fish; z: z-test estimate; ω^2: effect size estimate (omega squared); d: effect size estimate (Cohen's d); p: p-value after multiple comparison adjustment; statistically significant values are in bold.

(a)

(b)

(c)
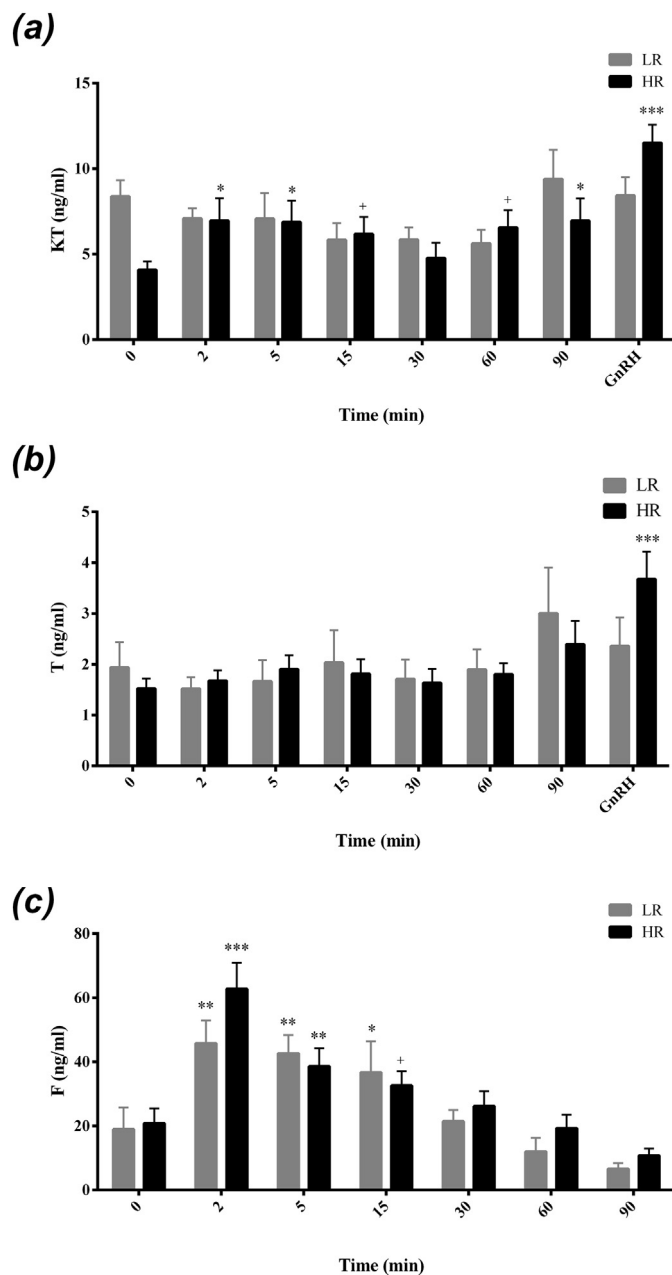
Fig. 4. Temporal pattern of the hormonal response to territorial intrusions for Low Responder (LR) and High Responder (HR) fish (see methods for details) in Social challenge experiment. Values are mean ± standard error of the mean (SEM). *(a)* 11-ketotestosterone (KT); *(b)* testosterone (T); *(c)* cortisol (F); [+] non-significant trend $p < 0.10$; * $p < 0.05$; ** $p < 0.01$; *** $p < 0.001$.

the second PCA score of the territorial intrusions ($r = -0.466$, $p = 0.039$, $n = 20$), suggesting that individuals with higher scores of exploratory behavior were less aggressive towards the neighbor.

3.2. Physiological challenge experiment

The levels of KT changed significantly with time ($F_{(5,67)} = 3.862$, $p = 0.004$, Table 3) but were not significantly affected by treatment with GnRH or control (saline) ($F_{(1,14)} = 0.462$, $p = 0.508$, Table 3). However, the interaction between these factors was significant ($F_{(5,67)} = 9.568$, $p < 0.0001$, Table 3). The levels of T changed significantly with time ($F_{(5,68)} = 7.851$, $p < 0.0001$, Table 3) but were not significantly affected by treatment ($F_{(1,14)} = 3.380$, $p = 0.087$, Table 3). The interaction between these factors was significant

($F_{(5,68)} = 11.529$, $p < 0.0001$, Table 3). Fish injected with GnRH significantly decreased KT, 2 min and 5 min after the injection and then significantly increased at 60 min, compared to the baseline (Table 3, Fig. 5a). For the control group, KT significantly decreased 15 min after the injection and remained below the baseline even after 60 min (Table 3, Fig. 5a). For T, there was a significant increase of T only for GnRH-treated group 30 min and 60 min time points (Table 3, Fig. 5b).

4. Discussion

Our results show that the androgen response to territorial intrusions varies between dominant males of the Mozambique tilapia and is related to their scope for response (i.e. the difference between baseline and maximum physiological levels for each fish). Individuals that have a lower scope for androgen response did not increase androgens after a territorial intrusion but were more aggressive and exploratory. Males with a higher scope for response have fewer aggressive and exploratory behaviors and exhibit two waves of KT response, one soon after the aggressive challenge (2–15 min) and other about an hour later (60–90 min). Moreover, subjecting fish to a GnRH challenge elicits an androgen increase 30–60 min after the injection.

In the case of the Mozambique tilapia, a polygynous species where males do not show parental care, the Challenge Hypothesis predicts a low androgen response to social interactions, when compared to species with other mating systems (Wingfield et al., 1990). We found that androgen responsiveness was rather variable, even though males had the same social rank, since baseline and physiological maximum levels differed largely between individuals. Therefore, males with KT baseline levels very close to their maximum (Low responder fish, LR) were not capable of significantly increasing androgen levels either to social or physiological challenges. Interestingly, other males (High responder fish, HR) had a higher scope for response and exhibited two peaks of KT levels, probably playing different roles. The quick response of KT may be related to the necessity to deal with the ongoing fight, regulating the male's behavior during the interaction (Marler et al., 2005). Another explanation for this short-term response can be a stress-provoked response to intrusion that has been described in acute stress events (e.g. androgen elevation 3 min after confinement in sockeye salmon, *Onchorhynchus nerka*: Kubokawa et al., 2001), which is confirmed by the elevated levels of cortisol soon after the social challenge. Several authors have proposed that fast androgen responses may enable swift and flexible behavioral responses to social challenges (Kempenaers et al., 2008; Oliveira, 2004). On the other hand, the delayed KT response suggests its involvement in the modulation of future interactions, possibly as a result of the outcome of the interaction (e.g. winner/loser effects) (Hsu et al., 2011; Oliveira et al., 2009; Oyegbile and Marler, 2005). From an overall perspective, we verified that KT increased in some individuals but decreased in others, hence these changes would cancel out each other at the population level. These results further support the idea that endocrine studies should focus on individual data rather than on the mean value of an heterogeneous group of individuals (Bennett, 1987; Williams, 2008). For instance, in bird breeding populations in the wild male T levels may vary 200-fold (e.g. blue tits, Kempenaers et al., 2008; black redstarts, *Phoenicurus ochruros*, Apfelbeck et al., 2013). Even within the same social status, animals can have distinct endocrine profiles, baseline and environment hormone reactions (e.g., Alcazar et al., 2016; Virgin and Sapolsky, 1992). Moreover, to compare traits or physiological measures obtained in different situations the same individuals should be used in within-subject designs (Bennett, 1987; Williams, 2008). In addition, not only the magnitude but also the temporal pattern of the androgen response can vary, which means that the variation of response with time for each individual is much more relevant than single 'snapshots' of the hormonal variation (Kempenaers et al., 2008).

Interestingly, our results also show that the scope for response is associated with body condition, with LR males exhibiting a higher body

Table 2

Statistical values for the differences between behavior of LR and HR fish in personality tests and over the territorial intrusions.

	Mean LR	Mean HR	t (df)	p	d
Open field test					
Distance moved in the centre zone	841.222	450.677	1.896 (18)	.074	0.834
Total distance moved	1714.852	824.268	2.095 (18)	**.050**	1.000
Total time in movement	221.362	114.180	1.994 (18)	.062	0.981
Territorial intrusions					
Total number of aggressive behaviors (males)	35.090	26.400	2.550 (18)	**.020**	1.237
Total time spent in aggressive behaviors (males)	203.119	179.333	2.067 (18)	.053	0.928
Total number of aggressive behaviors (intruder)	28.281	21.405	2.210 (18)	**.040**	1.080
Total time spent in aggressive behaviors (intruder)	171.867	152.838	1.736 (18)	.100	0.822
Total number of aggressive behaviors (neighbor)	6.810	4.995	1.839 (18)	.082	0.609
Total time spent in aggressive behaviors (neighbor)	31.252	26.495	0.339 (18)	.738	0.151
Total number of aggressive behaviors (females)	1.938	1.808	0.181 (18)	.858	0.091
Total time spent in aggressive behaviors (females)	3.267	1.854	0.470 (18)	.644	0.216
Total number of non-aggressive behaviors	1.457	1.764	−0.354 (18)	.727	0.160
Total time spent in non-aggressive behaviors	5.062	15.051	−1.231 (18)	.234	0.598
Total number of behaviors	38.486	29.972	2.518 (18)	**.021**	1.156

LR – Low responder fish; HR – High responder fish; t: t-test estimate; d: effect size estimate (Cohen's d); p: p-value; statistically significant values are in bold.

condition index than HR males. This finding suggests the occurrence of a trade-off between androgen responses and physical condition, such that individuals that have a low body condition (typically HR) are not able to maintain high levels of androgens, and thus sharply increase androgens when faced with a challenge, whereas, individuals with a good body condition (typically LR) keep their androgen levels high, and when challenged exhibit a low response. This finding is in agreement with previous work which has shown that keeping high levels of androgens is energetically costly, and is also associated to immune-suppression and oncogenic effects (Oliveira, 2004; Wingfield et al., 2001).

At the behavioral level, we found several behaviors to be quite variable between individuals but consistent within individuals. Regarding personality tests, behavior variables were repeatable only in the Open field (OF) test. This demonstrates that exploratory behavior is consistent in this species within a two months interval, even when subjecting males to repetitive behavioral and experimental manipulations. The other tests, Novel Object (NO), Mirror Test (MT) and Net Restraining (NR), seemed to be influenced by the underlying factors and could not be used to characterize consistent behavioral profiles. For

example, a lack of consistency across contexts (social vs isolation) of the NO test in this species has already been reported (Galhardo et al., 2012), while a lack of consistency across time has been found for other species (e.g., Gilthead seabream, *Sparus aurata*: (Castanheira et al., 2013). Supporting consistency of exploratory behavior and not of other traits in other taxa, are long-term personality studies carried in zebrafinches, *Taeniopygia guttata*, which found fearlessness and exploration the most repeatable traits within and across life phases, contrary to struggling rate (comparable test to the NR), aggression or boldness (David et al., 2012; Wuerz and Krüger, 2015).

In line with our results, temporal consistency in agonistic behavior has been previously found for other species (e.g., Australian lizard, *Egernia whitii*, While et al., 2010; bluefin killifish, *Lucania goodei*, McGhee and Travis, 2010; sheepshead swordtail, *Xiphophorus birchmanni*, Wilson et al., 2013). However, most of the behaviors, either aggressive or not, during the several sessions of territorial intrusions were found to be repeatable. Interestingly, a study in European seabass, *Dicentrarchus labrax*, performed coping screening tests in different contexts (isolation vs group) and repeated them within various time

Table 3

Effect of time and treatment (GnRH vs saline) on hormone levels (Physiological challenge experiment). Main effects (LMM), interactions, effect sizes and planned comparisons between the baseline and the other time points.

Comparisons	Main effects			Planned comparisons					
				GnRH group			Saline group		
	F	p	ω^2	z	p	d	z	p	d
KT			0.608						
Time	3.862	**.004**							
Treatment	0.462	.508							
Time × treatment	9.568	**< .0001**							
0 min vs 2 min				−2.170	**.050**	0.861	−0.833	.405	0.264
0 min vs 5 min				−3.152	**.005**	1.248	−1.956	.072	0.611
0 min vs 15 min				−1.637	.127	0.673	−2.198	**.050**	0.737
0 min vs 30 min				0.843	.405	0.335	−2.643	**.021**	0.842
0 min vs 60 min				3.188	**.005**	1.482	−3.533	**.004**	1.479
T			0.820						
Time	7.851	**< .0001**							
Treatment	3.380	.087							
Time × treatment	11.529	**< .0001**							
0 min vs 2 min				−0.425	.936	0.105	0.199	.936	0.057
0 min vs 5 min				−2.345	.055	0.682	−2.205	.055	0.184
0 min vs 15 min				0.723	.783	0.362	−0.071	.943	0.236
0 min vs 30 min				4.148	**.0002**	1.683	−2.266	.055	0.945
0 min vs 60 min				5.161	**< .0001**	1.774	−0.218	.936	0.054

11-ketotestosterone (KT); testosterone (T); z-test estimate; ω^2: effect size estimate (omega squared); d: effect size estimate (Cohen's d); p: p-value after multiple comparison adjustment; statistically significant values are in bold.
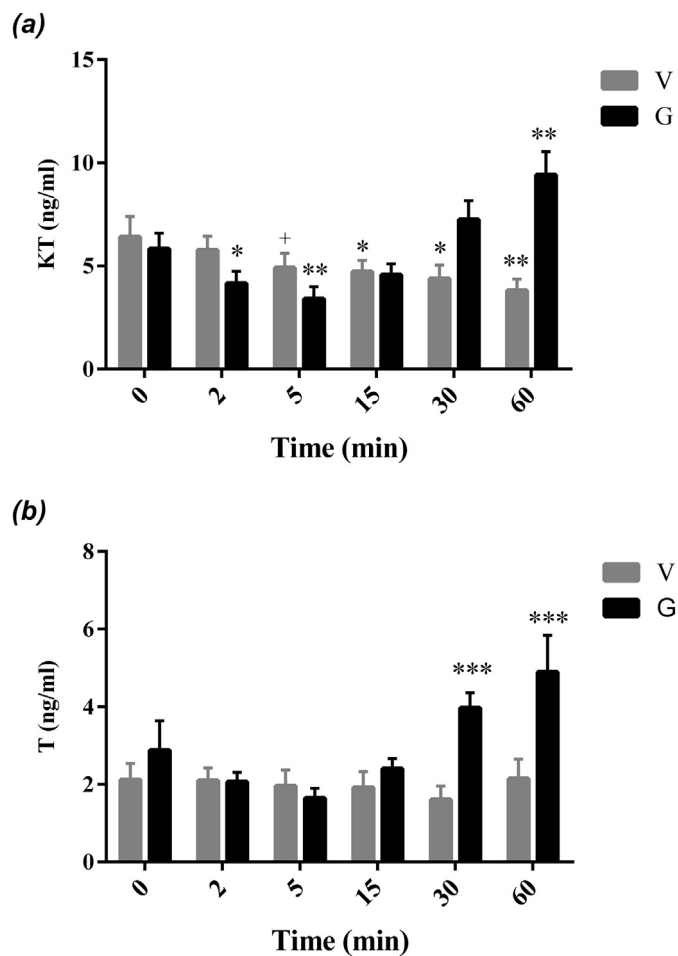
Fig. 5. Temporal pattern of the hormonal response of fish injected with vehicle (V) or with GnRH (G) in the Physiological challenge experiment. Values are mean ± standard error of the mean (SEM). *(a)* 11-ketotestosterone (KT); *(b)* testosterone (T); * p < 0.05; ** p < 0.01; *** p < 0.001.

intervals (up to 629 days) and found that, in opposition to individual tests, group-based tests were consistent across contexts and time (both short- and long-term) (Ferrari et al., 2015). Together these data suggest that social context promotes behavioral consistency at the individual level.

In our experiment we found a moderate negative correlation between aggressive behavior towards the intruder and exploration, that could be defined as a 'behavioral syndrome' (Bell, 2007; Sih et al., 2004) and could mean that the traits are regulated by a common neuroendocrine, genetic or neurobiological mechanism (Coppens et al., 2010; Sih et al., 2004).

The literature offers contradictory findings from several authors which attempted to explore the relationship between androgens and behavior, yet using baseline androgen levels (e.g. positive relation of exploration, boldness and aggression with T baseline levels in the mangrove rivulus, *Kryptolebias marmoratus*, Chang et al., 2012; positive relation between aggressive behavior and baseline T levels in male rats (Tryon Maze Dull S-3 rats), Schuurman, 1980; negative relation of exploration and boldness with T baseline levels in male great tits, *Parus major*, van Oers et al., 2011; negative relation of aggression with T baseline levels in male Australian lizards, *E. whitii*, While et al., 2010; no differences in T baseline levels between rats, *Rattus norvegicus*, selected for high or low levels of aggression, Everts et al., 1997). In the current study, individuals with a lower scope for KT response presented higher exploratory, activity, aggressiveness and body condition indices, in opposition to individuals with a higher scope of response. This set of

results links relative levels (baseline versus physiological maximum), rather than absolute levels, of KT to exploration, activity and male-male aggressive behavior.

Finally, it is important to mention that other factors not addressed in this study may also contribute to the observed inter-individual variation in androgen responsiveness to social interactions, such as variation in steroid metabolizing enzymes in target cells (Cornil et al., 2012; Roselli et al., 2009) or in the number, affinity and specificity of androgen receptors (e.g. androgen receptors vary as a function of the social environment, Fuxjager et al., 2010). Future studies accounting for these factors are worth exploring.

The results obtained for the stimulation of the HPG axis by treating animals with GnRH suggest that the two waves of KT response could be mediated by different physiological mechanisms. The late (90 min) response of androgens to social interactions seems to agree with the temporal response of the HPG axis, whereas the short-response does not. Research in mammals has confirmed the existence of a direct neural pathway responsible for the regulation of gonadal functions, including testosterone secretion (Mayerhofer, 2007; Selvage et al., 2006). The involvement of this pathway in the androgen response to social stimuli has never been explored. However, it is a mechanism, independent of the pituitary release of gonadotropins into circulation and its transport to the gonads, which seems compatible with the quick response we observed. In the Nile tilapia (*Oreochromis niloticus*), a close related species, nerve bundles have been identified in the testis close to Leydig cells (Nakamura and Nagahama, 1995), which may be controlling androgen release. Further studies on this question are needed to assess if the early and late androgen responses are mediated by direct neural pathways vs. HPG axis.

In summary, we found that the androgen response to social challenges varies between males depending on their scope for response, and when present it presents two peaks that seem to be regulated by different physiological mechanisms: an early response probably mediated by direct neural pathways followed by a late endocrine response mediated by the HPG axis. We suggest that determining the temporal pattern of the androgen response to social interactions and considering individual variability may be the key to understanding contradictory results of the Challenge Hypothesis.

Author contributions

ASF and RFO designed the experiment; ASF and AR performed behavioral experiments; ASF processed samples and analyzed the data; ASF and RFO wrote the paper.

Acknowledgements

Data availability statement

The datasets generated for this study are available on request to the corresponding author.

Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.yhbeh.2020.104755.

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