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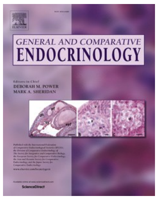

Research paper

Selection for winners impacts the endocrine system in the Siamese fighting fish

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ABSTRACT

In southeast Asia, males of the Siamese fighting fish, *Betta splendens*, have been selected across centuries for winning paired staged fights and previous work has shown that males from fighter strains are more aggressive than wild-types. This strong directional selection for winners is likely to have targeted aggression-related endocrine systems, and a comparison between fighter and wild-type strains can bring into evidence the key hormones implicated in aggression. Here, we compared the plasma levels of the androgen 11-ketotestosterone (KT) and of the corticosteroid cortisol (F) in F2 males of a fighter and a wild-type strain raised under similar laboratory conditions. We show that F was generally lower in fighter as compared with wild-type males, while no overall differences in KT levels were detected between strains. When presented with a mirror-induced aggressive challenge, post-fight levels of F increased but more significantly so in wild-type males, while KT increased in males of both strains. After the challenge, fighter males had higher levels of KT as compared with wild-type males, while the pattern for F was opposite. As compared with animals in social groups, wild-type males placed under social isolation had lower F levels, while KT decreased for fighters.

Taken together, this data suggests that while wild-type males responded to aggression with an increase in circulating levels of both androgens and corticosteroids, males selected for winning fights maintained a blunt F response, increasing only KT levels. These data agree with the hypothesis that a combination of high levels of androgens and low levels of corticosteroids is associated with high aggression. Overall, these results seem to indicate that selection for winning had a stronger impact in the hypothalamus-pituitary-interrenal axis than in the hypothalamus-pituitary–gonadal axis in *B. splendens*.

1. Introduction

The seminal work by Arnold A. Berthold (Berthold, 1849) with castration in chicks showed that “blood-born” secretions had a profound impact in the adult phenotype, including behavior. Since then, numerous studies have identified the chemical nature, sites of secretion, and action mechanisms of these molecules responsible for behavioral modulation, which we now know are steroid hormones (Nelson and Kriegsfeld, 2017). Consistent differences in adult behavior across groups, such as those observed between males and females for sexual or aggressive behavior, have been associated with organizational effects of sex hormones during development and with constitutive variation in hormones levels, in particular androgens of gonadal origin (McEwen and Milner, 2017). On the other hand, intra-individual variation in behavior, such as that observed seasonally or according to the dominance status of the animal, has been linked to fluctuations in hormone levels that translate changes in the physical or social environment into behavioral adjustments (Gonçalves et al., 2017).

A conceptual framework for both intra and inter-specific variation in aggression was proposed by John Wingfield and collaborators under the “Challenge Hypothesis” (Wingfield et al., 1990). This hypothesis, first developed for birds, states that androgen levels can fluctuate from a constitutive baseline observed during the non-reproductive period, to a breeding baseline responsible for the enhancement of mating and territorial behavior, and finally to a maximum physiological level, if animals are socially challenged. Under this hypothesis, androgens act as key translators of the social environment, adjusting physiology and behavior to environmental conditions and social context. Moreover, differences across species in territorial and aggressive behavior would also be explained by variations in androgen levels. Although this hypothesis has been generally confirmed for different vertebrate groups, including fish (Hirschenhauser et al., 2004; Hirschenhauser and

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Oliveira, 2006), there are also several studies that have failed to verify its assumptions. For example, while in monogamous birds androgens are expected to increase in response to an aggressive challenge, in several species this was not verified (e.g., Landys et al., 2007; van Duyse et al., 2004). Further, while data suggest an effect of peripherally secreted androgens in the regulation of aggression (Hirschenhauser and Oliveira, 2006), the mechanism through which androgens may affect the central nervous system and behavioral output remains largely unknown and several contradictory studies have been published, questioning the role of androgens as modulators of aggressive displays. For instance, castration failed to reduce male aggression in the Mozambique Tilapia *Oreochromis mossambicus*, in spite of a clear reduction in circulating androgen levels (Almeida et al., 2014). Similar results were suggested for the Siamese fighting fish *Betta splendens*, although in this case endocrine validation of the castration procedure was not possible (Weiss and Coughlin, 1979).

These findings have driven the search for alternative modulators of aggressive behavior. Corticosteroids, in particular, have been associated with the expression of aggression in several vertebrate species, although conflicting evidence also exists. Work in salmonids has shown that cortisol (F) administration (Gilmour et al., 2005) and high baseline F levels (Sloman et al., 2001) increases the probability of a fish to become a subordinate, suggesting an inverse relationship between circulating F levels and dominance behavior. On the contrary, F has been shown to increase after unresolved agonistic interactions in *O. mossambicus* (Félix et al., 2020) and *B. splendens* (Ramos et al., 2021), but the F pattern after a fight has been resolved, or after a change in dominance status, has not been investigated in these species.

An interaction between cortisol and androgens in the regulation of dominance behavior has been proposed for humans where androgens would promote dominance behavior only under low F levels, a hypothesis coined by Mehta and Josephs (2010) as the “dual-hormone hypothesis”. However, this hypothesis has so far not been experimentally investigated in non-humans and existing evidence in animals is again conflictual.

Clearly, the physiological mechanisms responsible for animals adjusting their aggressive behavior according to their past experience, environmental conditions, and social context are still poorly understood and further studies are needed.

The Siamese fighting fish *B. splendens* presents an excellent opportunity to test some of these hypotheses. The species has been domesticated for a period estimated to be of over 1000 years (Kwon et al., 2021) and, due to the naturally high level of aggression of males, used for cultural purposes in staged fights. In these contests, matched for size males are placed in a small container and fight until the dispute is resolved. The siblings of the winner male are bred for further selection while the loser batch is discarded. This long-term selection process for winners has originated “fighter” strains that are morphologically distinct and more aggressive than wild-type counterparts (Ramos and Gonçalves, 2019; Verbeek et al., 2007). A previous study comparing a wild-type and a fighter strain indeed suggests that the selection process has impacted the endocrine system, as water-borne F levels increased in response to an unfamiliar environment in wild-type but not in fighter males (Verbeek et al., 2008). However, the impact of selection for winners in androgens and in the context of aggression has so far not been investigated in this species.

Here, we compared the plasma levels of the androgen 11-ketotestosterone (KT) and of the corticosteroid F between a fighter and a wild-type strain of *B. splendens* raised under similar laboratory conditions. Variation in hormone levels were tested both in a social context, in isolation, and after an aggressive challenge. If indeed androgens underlie the differences in aggression reported for the tested strains, it was predicted that fighters should have either constitutively elevated androgen levels or a higher androgen responsiveness to an aggressive challenge. Considering the data available for fish, it was predicted that if corticosteroids also modulate aggression in this species, fighters should have constitutively lower levels of F or a lower F increase in response to a challenge. These two hypotheses are not mutually exclusive and indeed a combination of high levels or responsiveness for androgens and low for corticosteroids may underlie the expression of highly aggressive behavior in *B. splendens*.

2. Methods

2.1. Fish

The process of fish capture, mating and maintenance was the same as described in Ramos and Gonçalves (2019). Briefly, fighter fish were acquired from a reliable and authorized commercial fish farm from Bangkok, Thailand. A new fighter line was started at the lab, replicating a mating scheme commonly used by local breeders where sibling males of a winner are mated with sibling females from another winner. To perform that scheme, we used 12 sibling males from one breeder and 12 sibling females from another breeder (F0). From the result of the replicating matings, F1 males and females from different families were crossed to originate the F2 fish used in this study. Wild-types had been captured in Chiang Rai Province, Thailand, and were acquired from a local and authorized retailer. A mix group of 60 individuals (F0) was used to create a new population in the lab (F1). Ten pairs of F1 males and females from different families (different F0 parents) were mated to originate the F2 fish, with an approximately similar number of fish per family used in the experiments. Under laboratory conditions, the artificially selected traits associated with winning were probably under relaxed selection because there was no attempt to further select winners. The potential decrease in expression of the winning-related traits was minimized by limiting the initial genetic pool to two fighter families and by testing animals after only two generations.

The families were maintained under controlled laboratory conditions. The breeding tanks (50 W × 30D × 25H cm) only had shelters and aquatic plants to allow the female to hide and the water was conditioned with Indian almond leaf. The male was added to the tank and the female to a separate transparent box (10 × 10 × 10 cm) inside the larger tank. The male had only visual communication with the female. When a bubble nest was constructed by the male, the female was carefully released and spawning usually occurred within 24 h. After spawning, the female was removed from the tank and the male was allowed to provide parental care to the eggs and larvae for a period of 5 days. The male was then removed and the brood was divided after 1 month into similar tanks to achieve an approximate raising density of 1 fish per 3L. When fish were 2-month-old, an external filter was added to the tank. We used 8 +- 1 months old males of both strains for all experiments. Standard length (cm) of fish from the two strains used in the experiments did not differ (x ± S.D., fighter = 3.78 ± 0.02; wild-type = 3.42 ± 0.05). Sex-ratio in the stock tanks was approximately 1:1. Conditions in the stock and experimental tanks were similar, with temperature being kept at 28 ± 1 °C, the photoperiod set to 12:12 L:D and tank water supplied by a reverse osmosis system. Animals were fed once a day with a mixture of dry (tubifex worms and pellets from different brands) and live (adult *Artemia*) food.

2.2. Experimental setup

The impact of artificial selection for aggression in the endocrine system was assessed by comparing circulating plasma levels of F and KT between wild-type and fighter males kept in social groups and after a response to a mirror test. To minimize potential interindividual variability in endocrine levels before the mirror test, all fish were socially isolated and a control group of socially isolated fish that did not fight was also included. Thus, F and KT levels were compared between fish belonging to three groups: social group, social isolation, and mirror-fight (Fig. 1). On the same day, all males kept in the same stock tank were randomly assigned to each of the three treatments. For fish in social

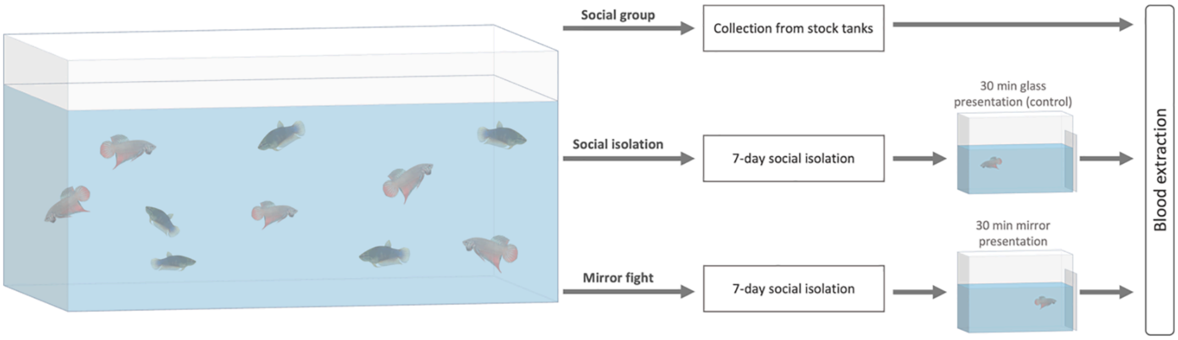

Fig. 1. Experimental design for measuring circulating levels of 11-ketotestosterone and cortisol from fish in social groups, after social isolation and after a mirror fight.

groups, males kept in stock tanks were captured and blood extracted for hormone assays (see below). Fish used for social isolation and mirror fights were collected from the stock tanks and placed in individual tanks (28 W × 14D × 20H cm; 4.5 L) without any visual contact with other fish. Tank walls were lined on the outside with white opaque acrylic plates. After seven days, fish assigned to the mirror-fight treatment were presented with a mirror placed adjacent to one of the shorter walls of the tank. The mirror test had a duration of 30 min and this stimulus has been previously demonstrated to induce a strong and equivalent aggressive and endocrine (KT, T and F) response to a live conspecific in this species (Ramos et al., 2021; Ramos and Gonçalves, 2019). Fish in social isolation were also used as control for the mirror treatment and were presented with a transparent glass instead of a mirror. The glass presentation did not induce any aggressive displays nor any visible change in the fish behavior. The tank presentation side for the mirror and glass was balanced. All fish fought the mirror throughout the 30 min test and all trials were considered valid.

Immediately after capture for males in the social groups, or at the end of each test for males presented with the mirror or glass, fish were anaesthetized with buffered MS222 (concentration 300 mg/L) and blood (app. 15–30 μl) was extracted from the caudal vein with a heparinized 30G syringe. The time between fish capture and blood extraction was below 8 min (average 5.2 ± 0.4 min). After extraction, fish were transferred to individual recovering tanks with aeration and clean water at 28 ± 1 °C. The blood samples were centrifuged at 7.5 rpm for 15 min, plasma was transferred to new clean tubes and stored at −20 °C until further analysis.

2.3. Hormonal analysis

In total 238 plasma samples from males were collected (N = 123 fighters; N = 115 wild-types), distributed across groups as follows: F, social group fighter N = 11, social group wild-type N = 12, social isolation fighter N = 24, social isolation wild-type N = 19, mirror fighter N = 16, mirror wild-type N = 11; KT, social group fighter N = 8, social group wild-type N = 8, social isolation fighter N = 40, social isolation wild-type N = 39, mirror fighter N = 24, mirror wild-type N = 26. KT and F were measured by competitive enzyme-linked immunosorbent assay (ELISA) kits (Cayman Chemical) following the manufacturer's instructions. Due to the low volume of plasma that can be collected from these fish, steroids were not extracted to reduce sample loss. For the same reason, androgen levels were only quantified for KT and not for T, as KT is the most potent androgen in fish (Borg, 1994) and the plasma dilution for the T ELISA (1:15) would preclude the quantification of F. A lack of interference in the assay of other immunoreactive molecules for this species and for these ELISA kits had been previously confirmed by serially diluting a plasma pool and comparing the slope with that of a standard curve (Ramos et al., 2021). All standards and samples were measured in duplicate with a dilution in the EIA buffer of 1:150 for KT and 1:20 for F. Duplicates from the same male were measured in the same assay. Average intra-assay coefficient of variation as determined from duplicates was of 2.97 % for KT and 3.53 % for F. Average inter-assay coefficients of variation were determined by adding two samples from two different plasma pools to each assay and were of 6.53 % for KT and 10.23 % for F.

2.4. Statistical analyses

A general linear model with factors strain (fighter and wild-type) and treatment (social group, social isolation, mirror fight) were used to compare hormone levels. Planned comparisons defined *a priori* were run to investigate differences between strains for each of the three treatments and differences within strains across treatments. Normality and homoscedasticity were assessed with Shapiro-Wilk's and Levene's tests, respectively. Data for F was log-transformed to comply with the homoscedasticity assumption. Correlation between F and KT hormone levels were tested with Person's correlation. All statistical analyses were run with R 4.1.0 (R Core Team, 2021).

2.5. Ethical note

All methods adhered to the ASAB/ABS ("Guidelines for the treatment of animals in behavioural research and teaching," 2012). The study followed the ethical guidelines enforced at the Institute of Science and Environment of the University of Saint Joseph and were approved by the Division of Animal Control and Inspection of the Civic and Municipal Affairs Bureau of Macao, license AL017/DICV/SIS/2016.

3. Results

3.1. Differences between strains

Selection for winners seems to have impacted constitutive levels of F. Plasma levels of this hormone were generally higher for wild-types than for fighters (wild-type, N = 42; fighter, N = 51, main effect of strain, $F_{(1,83)} = 27.674$, $p < 0.001$). Planned comparisons showed significant differences between strains for animals kept in social groups (wild-type, N = 12; fighter, N = 11, $p < 0.001$), in social isolation (wild-type, N = 19; fighter, N = 24, $p = 0.049$) and after the mirror challenge (wild-type, N = 11; fighter, N = 16, $p = 0.001$) (Fig. 2).

On the contrary, wild-type and fighter fish had similar levels of plasma KT (wild-type, N = 73; fighter, N = 72, main effect of strain, $F_{(1,139)} = 1.235$, $p = 0.268$). Nevertheless, planned comparisons revealed that fighter males had higher KT levels after the mirror fight as compared to wild-type males (wild-type, N = 26; fighter, N = 24, $p = 0.016$), while no difference was recorded in the other comparisons (social groups, wild-type, N = 8; fighter, N = 8, $p = 0.999$; social isolation, wild-type, N = 39; fighter, N = 40, $p = 0.626$).

It was possible to measure both KT and F for some fish. No correlation was found between the levels of the two hormones for fish kept in

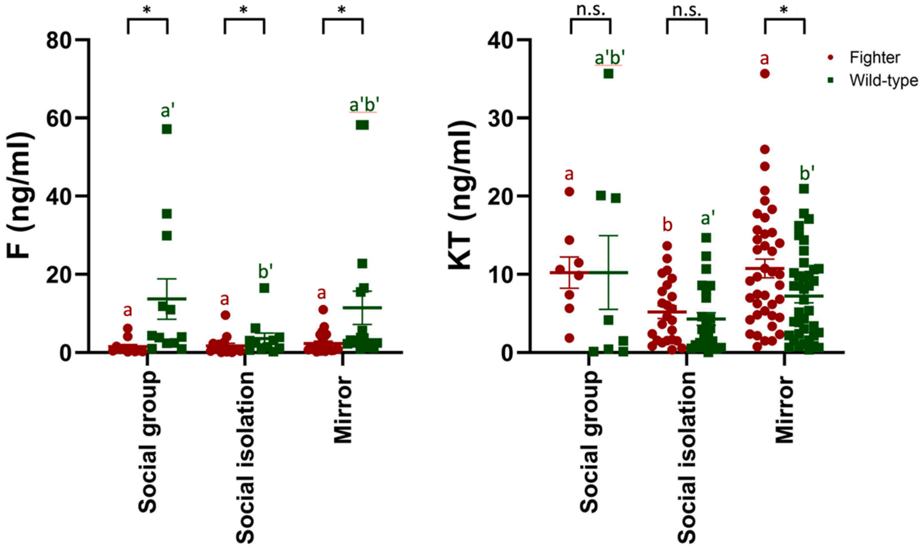

Fig. 2. Plasma levels of cortisol (F, left) and 11-ketotestosterone (KT, right) in wild-type and fighter males from social groups, kept in social isolation, or after a 30 min mirror fight. Individual values and mean ± S.E. are shown. * represent significant differences ($p < 0.05$) across strains for the same treatment. Different letters represent significant differences ($p < 0.05$) across treatments for the same strain. N for each group was as follows: cortisol, social group fighter N = 11, social group wild-type N = 12, social isolation fighter N = 24, social isolation wild-type N = 19, mirror fighter N = 16, mirror wild-type N = 11; 11-ketotestosterone, social group fighter N = 8, social group wild-type N = 8, social isolation fighter N = 40, social isolation wild-type N = 39, mirror fighter N = 24, mirror wild-type N = 26.

social groups (r = 0.003, N = 16, p = 0.990), in social isolation (r = 0.023, N = 26, p = 0.909) or after the mirror fight (r = −0.137, N = 42, p = 0.386).

3.2. *Response to an aggressive challenge and to social isolation*

Overall differences in plasma F levels between fish in social groups, in social isolation, or responding to the mirror were non-significant, although showing a trend (social groups, N = 23; social isolation, N = 43; mirror, N = 27, main effect of treatment, $F_{(2,83)} = 2.575$, p = 0.082). Planned comparisons between fish from the mirror and social isolation group show that post-fight F levels were elevated (p = 0.030), although specific comparisons between these two groups for wild-type (social isolation, N = 19; mirror, N = 11, p = 0.075) and fighter (social isolation, N = 24; mirror, N = 16, p = 0.202) males were non-significant. Interestingly, post-fight F levels were comparable to those measured in social groups (fighter, social groups, N = 11; mirror, N = 16, p = 0.316; wild-type, social groups, N = 12; mirror, N = 11, p = 0.605) (Fig. 2). Social isolation also had an impact in F levels, as given by the comparison between fish kept in social groups and in social isolation. Although there were no overall differences between fish from these two groups (social groups, N = 23; social isolation, N = 43, p = 0.106), within-strain comparisons showed that wild-type males kept in social isolation had reduced F levels (social groups, N = 12; social isolation, N = 19, p = 0.037), while no differences were found for fighter males (social groups, N = 11; social isolation, N = 24, p = 0.884).

Plasma KT levels differed significantly between groups (social groups, N = 16; social isolation, N = 79; mirror, N = 50, main effect of treatment, $F_{(2,139)} = 7.961$, $p < 0.001$). The mirror challenge induced a robust KT response (planned comparison between the mirror and social isolation control group, $p < 0.001$), which was observed for both wild-type (social isolation, N = 39; mirror, N = 26, p = 0.031) and fighter (social isolation, N = 40; mirror, N = 24, p = 0.001) males. After the mirror fight, KT levels were comparable to those measured from fish in social groups, both for fighter (social groups, N = 8; mirror, N = 24, p = 0.992) and wild-type (social groups, N = 8; mirror, N = 26, p = 0.973) males (Fig. 2). Social isolation also induced a decrease in KT levels (social groups, N = 16; social isolation, N = 79, p = 0.017), significant for fighter (social groups, N = 8; social isolation, N = 40, p = 0.040) but not for wild-type (social groups, N = 8; social isolation, N = 39, p = 0.185) males (Fig. 2).

4. Discussion

The main goal of the study was to investigate if long-term selection for winners had an impact in circulating levels of the androgen KT and of the corticosteroid F. Results suggest that the hypothalamus-pituitary-interrenal (HPI) axis was more impacted than the hypothalamus-pituitary–gonadal (HPG) axis.

Circulating levels of androgens were generally similar between the two strains. In social groups or in social isolation, KT levels were similar in fighter and wild-type males, suggesting that selection for winners did not have an impact in constitutive levels of KT. Nevertheless, when exposed with a mirror-simulated opponent, males of both strains robustly increased circulating KT levels, with fighter males attaining higher KT values than wild-type males. This might suggest that selection for winners enhanced the degree of the androgen response to an agonistic challenge. However, KT levels in fighter males after the mirror fight were equivalent to KT levels in wild-type males kept in social groups, showing that wild-type males were able to attain KT levels as high as fighter males. In social groups, males interact with other males and females and it is possible that this interaction raised androgen levels in wild-type males to levels comparable to those of fighter males after the mirror challenge. Differences in the mirror group between strains may be related to a faster KT time-response. In a study in *O. mossambicus*, KT was shown to peak at 2–15 min and 60–90 min after a short (3 min) social interaction (Félix et al., 2020). A similar study is underway to characterize the time-response in the two strains of *B. splendens* to test whether selection for winners has driven a faster androgen response. Alternatively, the mirror stimuli may have been more salient to fighter than wild-type males. The mirror test has been shown to produce an androgen response equivalent to a live conspecific in *B. splendens* (Ramos et al., 2021), but that study was performed with F2 fish from a cross between the two strains here used and possible differences between the parental strains in the endocrine response to different aggression-eliciting stimuli were not investigated.

The increase in androgen levels after the mirror-challenge is corroborated by a previous study in *B. splendens* males where it was described a clear post-fight surge in both T and KT levels after a mirror or live conspecific interaction (Ramos et al., 2021). They contrast, however, with a previous work with males of this species that failed to find a significant variation in water-borne KT levels after an interaction with a conspecific behind a transparent partition (Dzieweczynski et al., 2006). Possible reasons for this discrepancy are discussed in Ramos et al. (2021) and relate to methodological issues of measuring this hormone in water in the Dzieweczynski et al. (2006) study. In addition to these three studies, other experiments performed in our lab with alternative stimuli (video playback of a male, a male behind a one-way mirror) have also confirmed a robust androgen increase after a fight, even if unresolved, in male *B. splendens* (unpublished data).

One of the assumptions of the challenge hypothesis is that androgen levels are the result of male-male agonistic interactions (Wingfield et al., 1990). However, in different vertebrate taxa there are cases in which androgen levels fail to increase in response to an aggressive challenge (e.g., Baird et al., 2014; Moore et al., 2004; Ros et al., 2014), questioning the validity of this assumption. More recently, Goymann et al. (2019) proposed the "challenge hypothesis 2.0" according to which male–female interactions, rather than male-male interactions, would be more relevant in mediating rapid changes in androgen levels. In the present study, males sampled from social groups could interact with both males and females. However, males in social groups do not build nests and courtship displays towards females are also rare, while both intra and intersexual agonistic interactions are frequent (personal observations). The fact that androgen levels decreased with social isolation, as previously shown for territorial males of *O. mossambicus* (Galhardo and Oliveira, 2014), and that after the mirror fight KT levels were similar to those recorded in social groups for males of both strains, suggests that indeed male-male aggressive interactions are the key determinant of circulating androgen levels in *B. splendens*, in support of the challenge hypothesis. Nevertheless, comparing the relative KT response to male-male and male–female interactions would be relevant to confirm this hypothesis.

The follow up question after confirming that androgens increase in response to an aggressive challenge is then on the functional role of this increase. The rational of the challenge hypothesis is that androgens increase above the physiological level needed for reproduction in response to a social challenge to facilitate the expression of mate-guarding behaviors (in species with paternal care) and male-male aggression (Wingfield et al., 1990). This view is partially supported by a study where high dosage, but not low dosage, of T administration for 15 days increased aggressive displays in *B. splendens* (Forsatkar et al., 2013) and by a meta-analysis where exogenous androgen administration was confirmed to enhance aggressive behavior in fish (Hirschenhauser and Oliveira, 2006). However, no correlation between the frequency and duration of aggressive behaviors and post-fight androgen levels was recorded for male *B. splendens* in the Ramos et al. (2021) study. Further, castration, which is expected to reduce circulating androgen levels and prevent the mounting of a significant androgen response, failed to decrease aggression in *B. splendens* (Weiss and Coughlin, 1979) and in *O. mossambicus* (Almeida et al., 2014). The absence of difference between the fighter and wild-type males from social groups and in social isolation in KT levels further supports the view that peripheral androgen synthesis was not targeted by the selection process for winners, although the differences between strains observed for the mirror group do not allow to completely discard the hypothesis of an enhanced KT response in fighters that facilitated aggression. Future controlled experiments manipulating androgen levels may further test this hypothesis. In addition, the androgen system may have been targeted by the selection process at other levels, as for example by changing androgen receptor density or central androgen synthesis, for which comparing wild-type and fighter strains also for these parameters would be relevant.

In contrast to the results obtained for KT, levels of F were elevated in wild-type males in all experimental groups, as compared to fighter males. These results support the hypothesis that the selection process for winners lowered constitutive levels of F and its responsiveness to an agonistic challenge, enhancing aggression. In fact, in the rainbow trout *Oncorhynchus mykiss*, lines selected for low F responsiveness to stressors are more aggressive than lines selected for high F responsiveness (Øverli et al., 2004), with low F fish attaining more frequently a dominant status than high F fish (Pottinger and Carrick, 2001). Further, long-term, but not short-term, F administration inhibits aggression in the same species (Øverli et al., 2002).

Data here reported for F, however, only partially agrees with a previous study comparing a fighter and a wild-type strain of *B. splendens*. In that study, water-borne F levels of socially isolated males did not seem to differ between strains (Verbeek et al., 2008). Nevertheless, while males of both strains responded with an elevation of F to a confinement test, only wild-type males increase F levels in response to an unfamiliar environment, suggesting a higher stress reactivity of wild-type fish (Verbeek et al., 2008), and mimicking the results here obtained for the aggression test. Care should be taken in the interpretation of these results as measuring steroids from water needs to be carefully validated (Scott et al., 2008) and a correlation between water and plasma F levels in this species has still not been demonstrated.

Also, F levels were generally elevated after the mirror fight, as previously demonstrated for *B. splendens* (Ramos et al., 2021) and *O. mossambicus* (Félix et al., 2020). Similarly to what was described for androgens, post-fight F levels were also uncorrelated with aggression or activity levels in the Ramos et al. (2021) study. In the rainbow trout, F increases rapidly after a staged fight both in losers and in winners, but rapidly decays in future dominant fish while continues to increase in subordinates (Øvrli et al., 1999). Thus, one possibility is that short-term F increases with fight behavior regardless of fight outcome but that high long-term, or chronic, F elevation is associated with a reduction in aggressive behavior in *B. splendens*. While strain-specific post-fight increase in F levels were not significant, there was a general trend for F levels to be more elevated in wild-type ($p = 0.075$) than in fighter males ($p = 0.202$). This may suggest that selection for winners has favored a post-fight corticosteroid dominant profile, with a blunted F response to aggression. Experiments in fighter and wild-type strains manipulating pre-fight F levels and assessing the long-term impact of changes in dominance status in F levels are needed to better understand the possible impact of this hormone in the expression of aggressive displays and dominance behavior.

The possibility that overall variation in F levels between males of the two strains is unrelated to differences in aggression should also be considered. For instance, high levels of F in wild-type fish may reflect a much shorter process of domestication and habituation to captivity as compared with fighter males. Domestication in fish has been proposed to be associated with reduced activity of the HPI axis in response to stressors, although impacts in baseline F levels are less clear (Awata et al., 2011; Douxfils et al., 2011; Mazur and Iwama, 1993; Milla et al., 2021). Domestication in this species is thought to have commenced over 1000 years ago (Kwon et al., 2021) and although we do not have information about the domestication process of the fighter line used in this study, it has been under domestication for a much longer period than the used wild-type strain. Still, both strains were acquired at the same time to establish the laboratory lines and were under the same breeding and maintenance conditions for two generations in order to minimize differences related to response to captivity.

Taken together, the overall similar androgen pattern in males of the two strains, and the lower levels of F in fighters and a trend for a blunted F responsiveness to aggression and to social isolation in these males, supports the hypothesis that selection for winners had a stronger impact in the HPI axis than in the HPG axis. These results suggest that variation in circulating levels of corticosteroids, rather than in androgens, could mediate differences in aggression, and maybe in dominance, in this species. This "corticosteroid-mediated dominance hypothesis" is supported by previous work in several vertebrate species. For example, extreme aggression received by adult rhesus macaques *Macaca mulatta* correlate with F levels, which in turn are inversely correlated with dominance rank (Feng et al., 2016). In a fish example, subordinates of *O. mykiss* have sustainably elevated F levels, suggesting that they are under "chronic stress", probably driven by aggression received from dominant fish (Gilmour et al., 2005). In agreement, levels of F were lower in socially isolated wild-type males as compared with fish kept in social groups while no impact was recorded for fighter males. These results for wild-type males parallel those reported for zebrafish where chronic (6 months) social isolation reduced F levels (Shams et al., 2017). This may reflect a pattern whereby selection for winners has blunted a possible corticosteroid response from social interactions mediating subordinate behavior. Addressing the impact of winning/losing and of a change in social status in hormone levels in both wild-type and fighter strains will be needed to confirm this hypothesis.

Further, an interaction between androgens and corticosteroids also seems possible and may explain both inter and intrasexual variation in aggression. Under this "dual-hormone hypothesis", first proposed for humans, high levels of androgens and low levels of corticosteroids modulate high aggression levels (Mehta and Josephs, 2010), explaining why males generally express higher physical aggression levels than females and why dominants have generally lower corticosteroid levels than subordinates. Although this hypothesis has so far not been tested in animals, fish seem to be ideal candidates for this purpose as it is possible to independently manipulate the HPI and HPG axes using pharmacological (e.g., administration of exogenous agonist and antagonists of androgens and corticosteroids), surgical (e.g., gonadectomy) and genetic (e.g., gene knockouts for androgen and corticosteroid receptors) methods.

In conclusion, the data supports a more pronounced impact of selection for winners in the HPI axis than in the HPG axis, opening a number of testable hypotheses on the role of androgens and corticosteroids as modulators of aggression in *B. splendens.*

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