Hormones and Behavior 132 (2021) 104991

Contents lists available at ScienceDirect

Hormones and Behavior

journal homepage: www.elsevier.com/locate/yhbeh

Androgens and corticosteroids increase in response to mirror images and interacting conspecifics in males of the Siamese fighting fish *Betta splendens*

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ARTICLE INFO

Keywords:
Aggression
Androgens
Betta splendens
Corticosteroids
Live conspecific test
Mirror test

ABSTRACT

The role of hormones as modulators of aggressive behavior in fish remains poorly understood. Androgens and corticosteroids, in particular, have been associated with aggressive behavior in fish but it is still not clear if animals adjust the secretion of these hormones to regulate behavior during ongoing fights, in response to fight outcomes in order to adjust aggressive behavior in subsequent fights, or both. With its stereotyped displays and high aggression levels, the Siamese fighting fish *Betta splendens* is an excellent model to investigate this question. Here, we compared the behavioral and endocrine response of male *B. splendens* to fights where there is no winner or loser by presenting them with a size-matched live interacting conspecific behind a transparent partition or with a mirror image. The aggressive response started with threat displays that were overall similar in frequency and duration towards both types of stimuli. Fights transitioned to overt attacks and interacting with a live conspecific elicited a higher frequency of attempted bites and head hits, as compared with the mirror image. There was a pronounced increase in plasma androgens (11-ketotestosterone and testosterone) and corticosteroids (cortisol) levels in response to the aggression challenge, independent of stimulus type. Post-fight intra-group levels of these hormones did not correlate with measures of physical activity or aggressive behavior. A linear discriminant analysis including all behavioral and endocrine data was a poor classifier of fish from the conspecific and mirror trials, showing that overall the behavioral and endocrine response to mirror images and conspecifics was similar. The results show that fight resolution is not necessary to induce an evident increase in peripheral levels of androgens and corticosteroids in *B. splendens*. However, the function of these hormones during present and future aggressive contests remains to be clarified.

1. Introduction

The study of aggression was key to Darwin's theory of speciation by natural selection (Darwin, 1859) and has a long history within the fields of ethology and behavioral endocrinology. Understanding how animals decide when to initiate or escalate a fight or when to avoid or flee from a conflict is relevant because disputes over finite resources can have direct (e.g., access to mates) and indirect (e.g., access to food) fitness consequences (Parker, 1974). However, in spite of significant progress made in the past decades, the key physiological mechanisms and genetic pathways underlying intra and interspecific variation in aggressive behavior remain poorly known.

Fish show a remarkable variation in social systems and behavior and have a relatively well conserved organization of the neuroendocrine system, as compared with mammals (Oliveira and Gonçalves, 2008), making them promising models for the study of aggression. As in other vertebrates, hormones, and in particular androgens, have long been identified as key modulators of fish aggressive behavior, explaining why aggression is usually higher in males than in females, peaks in tandem with peripheral levels of androgens (e.g., during the breeding season), and is higher in dominant fish (Gonçalves et al., 2017). Further, evidence has been accumulating that decisions taken in an agonistic context are partly influenced by previous winning/losing experiences and that androgens modulate these decisions (Oliveira et al., 2009). Under this hypothesis, variation in peripheral androgen levels would translate previous fight outcomes, adjusting aggressive behavior according to context. However, conflicting evidence on the role of androgens as modulators of fish aggression also exists. For example, castration, with concomitant suppression of gonadal androgen secretion, failed to decrease aggression in the Mozambique tilapia,

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https://doi.org/10.1016/j.yhbeh.2021.104991
Received 4 January 2021; Received in revised form 24 April 2021; Accepted 24 April 2021

Oreochromis mossambicus (Almeida et al., 2014), and in the Siamese fighting fish, *Betta splendens* (Weiss and Coughlin, 1979). Exogenous administration of androgens has also produced conflicting results, increasing aggression in some studies (e.g., (Forsatkar et al., 2013)) but not in others (Kindler et al., 1991). Also, while in some species androgens increase only after the resolution of fights (Oliveira et al., 2005), in others they rise even in unresolved contests (e.g., Desjardins and Fernald, 2010), questioning a general role of androgens as translators of winning/losing experiences.

These contradictory results have driven the search for other possible modulators of aggressive behavior in fish and corticosteroids, in particular, have been receiving some attention. In general, higher levels of cortisol (F) seem to be associated with a subordinate status in fish. For example, rainbow trout, *Oncorhynchus mykiss*, treated with F were more likely to become subordinates in paired encounters with smaller untreated opponents (Gilmour, 2005) and, also in salmonids, high baseline F levels seemed to increase the likelihood of fish becoming a subordinate (Sloman et al., 2001). Further, long-term (48 h), but not short-term (1 h), F treatment inhibits aggressive behavior in *O. mykiss* (Øverli et al., 2002). On the contrary, F has been shown to rise after agonistic interactions in *O. mossambicus* (Félix et al., 2020). An interaction between androgens and corticosteroids has been suggested under the "dual-hormone hypothesis", first proposed for humans, according to which the positive effects of androgens on aggression would only occur under low F levels (Mehta and Josephs, 2010). However, until date this hypothesis has not been directly tested in fish and it is also not addressed in the current study.

When studying the physiological mechanisms of fish aggression under laboratory settings, one important technical aspect to address is how to test and measure behavior. Different tests have been adopted for this purpose, including the use of live fights, interactions across partitions, presentation of 2D and 3D models, videos or mirror images, and even robots (e.g., Romano et al., 2017; Way et al., 2015). The choice of test depends on a number of factors, such as the study species and the objectives of the study. For example, live fights provide a dynamic interaction between opponents with agonistic displays being modulated by the feedback received and a resolution of the fight is possible. Models or video playbacks deliver always the same feedback, allowing for more standardized test conditions but without fights being decided. The mirror test has been widely used to trigger aggression in fish as most species will attack their own image (e.g., Balzarini et al., 2014). Feedback received is phase-locked with the behavior of the focal fish and fights remain unresolved in this test. However, the use of mirrors to test aggression in fish has received some criticism as the behavioral (Balzarini et al., 2014), endocrine (Oliveira et al., 2005) and brain transcriptomic (Desjardins and Fernald, 2010) responses may differ to those of live conspecifics. Further, it has been recently suggested that some fish species may be able to self-recognize in mirror images (Khoda et al., 2018, but see de Waal, 2019). It is thus important to validate species-specific aggression tests by measuring and comparing the behavioral and physiological responses to different test types.

The Siamese fighting fish has been widely used to study aggression because of the highly stereotyped agonistic displays performed by males (Simpson, 1968). In nature, males establish territories from where they attract females with courtship displays and where they build and fiercely defend a bubble nest where eggs are deposited after mating (Jaroensutasinee and Jaroensutansinee, 2001). The social modulation of aggressive displays is complex and males have been found to adjust their behavior according to the presence and type of an audience (Doutrelant et al., 2001; Dzieweczynski et al., 2006; Matos and Mcgregor, 2002) and to be able to extract relevant information from watching fights (McGregor et al., 2001; Oliveira et al., 1998). Further, due to its high aggression levels, the species has been selected across centuries in Southeast Asia for cultural purposes where paired-fights between males are staged, originating "fighter" lines, more aggressive than wild-types (Ramos and Gonçalves, 2019; Verbeek et al., 2007). These characteristics make *B. splendens* a very interesting model for the study of the mechanisms of aggression in fish, facilitated by the recent sequencing of its genome (Fan et al., 2018). Different types of tests have been used to study aggression in this species, including live fights (e.g., Vu et al., 2020), opponents behind partitions (e.g., Oliveira et al., 1998), video playbacks (e.g., Dzieweczynski et al., 2014), 3D models (e.g., Halperin et al., 1992), robots (e.g., Romano et al., 2019), and mirrors (e.g., Takeuchi et al., 2010). Although a few studies in *B. splendens* have compared the behavioral response to different types of stimuli (e.g., Craft et al., 2003; Ramos and Gonçalves, 2019), this is the first study investigating differences in the endocrine response towards two aggression-eliciting stimuli in this species.

Here, we presented males of the Siamese fighting fish with a size-matched interacting conspecific opponent or a mirror image and compared their behavioral and post-fight plasma levels of the androgens 11-ketotestosterone (KT) and testosterone (T) and of the corticosteroid F. As both types of stimuli provide "matched" fights where there is no winner or loser, hormone levels were expected to differ from controls if they play a role during ongoing fights.

2. Methods

2.1. Fish

All subjects used in this experiment were 12-month-old *B. splendens* males from the F1 generation of a cross between a wild-type and a fighter strain (see Ramos and Gonçalves, 2019 for details on the parental lines). These two strains have been previously shown to differ in aggression, with fighters being more aggressive than wild-types (Ramos and Gonçalves, 2019). Testing the F1 progeny from these strains allowed integrating genetic variation within the species that may underlie differences in the endocrine and behavioral response to an aggression challenge. Fish originated from five batches from different couples and were raised in mixed-sex groups in 50 W X 30D X 25H cm tanks at a density of 50 fish per tank. Animals were isolated at ten months old into 9 W X 9D X 20H cm tanks, containing a small ceramic shelter, without visual contact with other conspecifics. Fish were fed twice a day with newly hatched artemia for the first four weeks post-hatching and then transitioned to commercial dry food (Golden pearl diet) in the morning and live food (adult artemia) in the afternoon, except on the day of the experiments, when no food was given. Conditions in the stock, isolation, and experimental tanks were similar, with temperature being kept at 28 ± 1 °C, the photoperiod set to 12 L:12D, and tank water supplied by a reverse osmosis (RO) system. For stock and isolation only, RO water was further conditioned with Indian almond tree leaves and salinity kept at 4 ± 1 g/L.

2.2. Experimental setup

Fish were randomly assigned to three treatments (control, conspecific and mirror) and did not differ in weight (W) or standard length (SL) between groups (One-way ANOVA, W, $F_{(2,33)} = 1.212$, $p = 0.310$; SL, $F_{(2,33)} = 2.282$, $p = 0.118$). Fish used as the conspecific stimuli were matched for size (SL, C.V. < 5%) and were not used as focal animals. One week prior to the beginning of the experiment, fish were transferred to new individual tanks of the same dimensions as the experimental tanks (25 W × 12.5 D × 20H cm for the focal fish; 12.5 W × 8 D × 20H cm for the conspecific stimuli fish) and netted and released back into their tank on a daily basis to habituate to handling.

The experiment was run inside a test box of 40 W × 49 D × 45H cm with white walls containing the tank of the focal fish and either the tank of the stimuli conspecific, a mirror or an empty tank (Fig. 1). The tank of the stimuli conspecific was smaller than the tank of the focal fish to promote interaction between the two males and to better match the stimuli provided by the mirror. Illumination was provided by a diffuse LED strip. An opaque smart screen that becomes transparent when

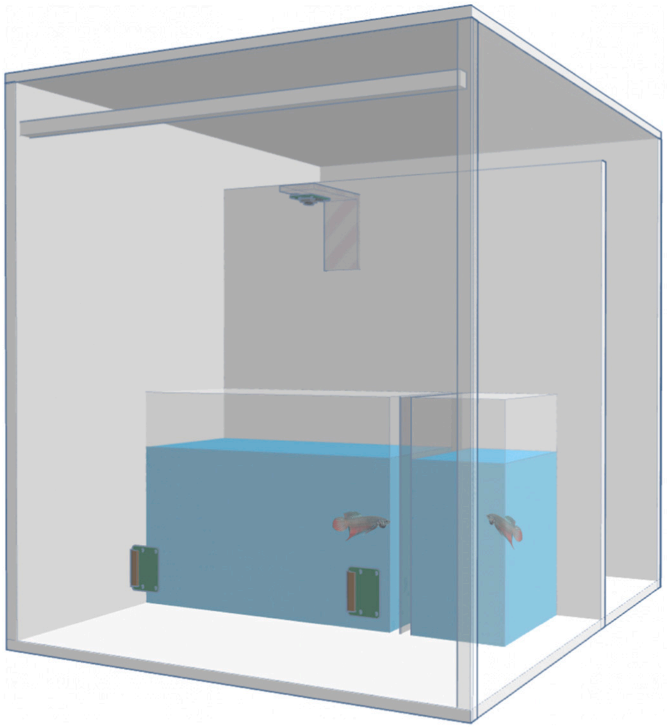

Fig. 1. Test box used in the experimental setup. The focal fish (larger tank) was exposed to a live conspecific (smaller tank) or, alternatively, to a mirror or an empty tank (not depicted). An opaque smart screen that becomes transparent when activated was placed between the focal fish tank and the stimuli and turned on 30 min after introducing the fish in the box to start the challenge. Behavior of the focal fish was recorded with a side and top camera and of the conspecific fish with a side camera. Illumination was provided by a diffuse LED strip.

activated separated the focal fish from the stimuli side. The behavior of the focal fish was recorded with one side and one top Raspberry Pi camera module (V2), and of the stimuli conspecific fish with a similar side camera, all at a resolution of 1280 × 720 px and 40 fps. Each camera was connected to an independent Raspberry Pi board (V3).

After, fish were transferred from their individual tanks to the test tanks, the cameras activated, and a 30 min habituation period was given. At minute 30, the smart screen was automatically activated to start the experiment. Observations had a duration of 30 min and the experimental tanks were replaced between trials.

For the focal fish, information from the two cameras was combined to extract, with an in-house script developed in Python and OpenCV, variables related with 3D position in the tank, including time spent within 5 cm of the stimuli and within 4 cm of the surface, head orientation and distance travelled. Fish darken their bodies when displaying and a measure of color change was obtained by comparing the average grayscale value (from 0 - black, to 255 - white) of a central area of the fish blob during the acclimation and test periods, excluding the first and last 5 min of each phase (5 to 25 min and 35 to 55 min for the acclimation and test phases, respectively). For this, all frames where the fish was lateral to the side camera (i.e. ±20°, as determined from the top camera coordinates) were selected and the average grayscale value of a square with 16 × 16 pixels, centered on the fish blob, obtained (Supplementary Fig. 1). The delta of the difference in average body grayscale between the acclimation and test phase was used as the measure of color change and compared between treatments. Aggressive behaviors of both the focal and stimuli fish were manually scored with Boris software (V. 7.9.19 for Mac, Friard and Gamba, 2016) (http://www.boris.unito.it/) and included: duration of opercular displays (also called gill cover erection), duration of unpaired fins distension, frequency of caudal swings and frequency of attempted bites/mouth hits (Simpson, 1968; Table 1). Attempted bites were not easy to discriminate from mouth hits in the glass and were considered together. Charges, a rapid swim towards the stimuli, were only displayed by two of the focal fish in each of the aggression-eliciting groups and were not included in the analysis. In labyrinth fish, the frequency of surface air breathing correlates with oxygen consumption during fights and provides a measure of metabolic effort (Alton et al., 2013). Accordingly, the frequency of surface air breathing was recorded.

At the end of the observation, focal fish were immediately removed from the tank, anaesthetized with buffered MS222 (concentration 300 mg/L) and blood (app. 15–30 μ) was extracted from the caudal vein with a heparinized 27G syringe. After the procedure, individuals were transferred to individual recovering tanks with aeration and clean water. The blood samples were centrifuged for 15 min, plasma was transferred to new clean tubes and stored at −20 °C until further analysis.

2.3. Hormone analysis

Plasma levels of KT, T and F were measured with competitive enzyme-linked immunosorbent assay (ELISA) kits (Cayman Chemical) following the manufacturer's instructions. Due to the small volume of plasma that can be extracted from these fish, and to the objective of measuring the three hormones in all fish, steroids were not extracted. The lack of interference in the assay of other immunoreactive molecules was confirmed by pooling small volumes of plasma from each fish, serially diluting the pool and comparing the slope with that of a standard curve (ANCOVA: KT, $F_{(1,12)} = 1.419$, $p = 0.257$; T, $F_{(1,12)} = 0.017$, $p = 0.899$; F, $F_{(1,12)} = 0.649$, $p = 0.436$). All standards and samples were measured in duplicate with a dilution in the EIA buffer of 1:150 for KT, 1:15 for T, and 1:20 for F. Experimental samples were measured in the same assay and the intra-assay coefficient of variation, calculated from the sample duplicates, was of 3.56% for KT, 4.46% for T and 2.87% for F.

2.4. Data analysis

Differences between groups in variables related with hormone levels and with activity and position in the tank were tested with one-way ANOVAs with factor treatment (control, conspecific and mirror) followed by post-hoc Tukey tests to assess differences between groups. Differences in aggressive behaviors between the live conspecific and mirror groups were tested with unpaired *t*-tests as aggression was not displayed during control trials. Normality and homocedasticity were confirmed with Shapiro-Wilk's and Levene's tests, respectively. Some variables were either log- or squared-transformed to comply with parametric assumptions. Considering the relatively low number of comparisons made, and following the recommendations of Nakagawa (2004), corrections for multiple comparisons were not applied and effect sizes are reported. Following the recommendations of Lakens (2013),

Table 1
Description of the aggressive and activity behaviors quantified.

Behavior	Description
Threat behaviors	
Opercular displays	Opens the opercula and branchiostegal membrane.
Unpaired fins extension	Distends the unpaired fins.
Caudal swing	Waves the caudal fin towards the opponent side.
Attack behaviors	
Bites/mouth hits	Sharply opens and closes the mouth to bite the opponent with physical contact with the partition/hits the partition with the mouth closed.
Activity behaviors	
Air breathing	Swims to the surface to engulf air.

omega squared estimates are provided for ANOVAs while for pair-wise comparisons Cohen's d were calculated.

To test if fish could be correctly assigned to their respective groups using information contained in the measured behavioral and endocrine variables, a linear discriminant analysis (LDA) was run. Due to the nature of the measured data, high collinearity and violation of parametric assumptions of variables were predicted and the scores of a principal component analysis (PCA) that included all behavioral and endocrine data were used as input variables for the LDA.

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 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. Behavior

Both the live conspecific and the mirror image induced an aggressive response from all focal fish within the first seconds after the trial was initiated. Without exception, the first response consisted of a frontal display with the opening of the opercula and distension of the unpaired fins, followed by the darkening of the body (Supplementary Fig. 1).

In spite of spending on average over 90% of the time close to the mirror or live conspecific, the distance travelled by fish during the aggression trials was twofold higher as compared to controls (Fig. 2, Table 2). Accordingly, the frequency of air breathing, an indicator of metabolic activity (Alton et al., 2013), increased fourfold when displaying aggression (Table 2) and was positively correlated with the distance travelled (Pearson's correlation, $r = 0.473$, $N = 22$, $p = 0.026$) and with the total time distending the fins ($r = 0.478$, $N = 23$, $p = 0.021$) but not with the time spent with the opercula open, frequency of caudal swings or frequency of bites ($p > 0.067$).

The conspecific and mirror image induced a similar metabolic effort, as inferred from the comparable frequency of air breathing, time spent close to the surface and total distance travelled (Table 2). Focal fish also spent an equivalent proportion of time close to the conspecific and mirror side (Table 2).

The darkening of the body was pronounced during the aggression trials, as compared with controls, but similar when reacting to the conspecific or mirror image (Fig. 3). Likewise, the time spent with open opercula and distended unpaired fins and the frequency of caudal swings did not differ between the mirror and conspecific groups (Table 3).

Approximately 8–9 min into the challenge, the frequency of threat displays decreased while overt aggression, as inferred from the frequency of bites/head hits in the glass, increased (Fig. 4). There was no difference in the latency of the first bite/head hit between the mirror and conspecific treatment (t-test, $t = -1.799$, d.f. $= 16$, $p = 0.091$), suggesting a similar temporal pattern of the transition from the threat to the attack phase in the two groups of fish. However, the frequency of bites/head hits was overall higher towards the live opponent (Table 3).

When interacting with the conspecific, the behavior of the focal fish is expected to depend on the feedback received from the opponent. However, no correlation was found between the frequency of air breathing, time spent with open opercula, time spent with distended fins or frequency of bites of the two opponents ($p > 0.295$). On the contrary, the frequency of caudal swings, a behavior that is often displayed by the two fish simultaneously, was highly correlated ($r = 0.915$, $N = 10$, $p < 0.001$).

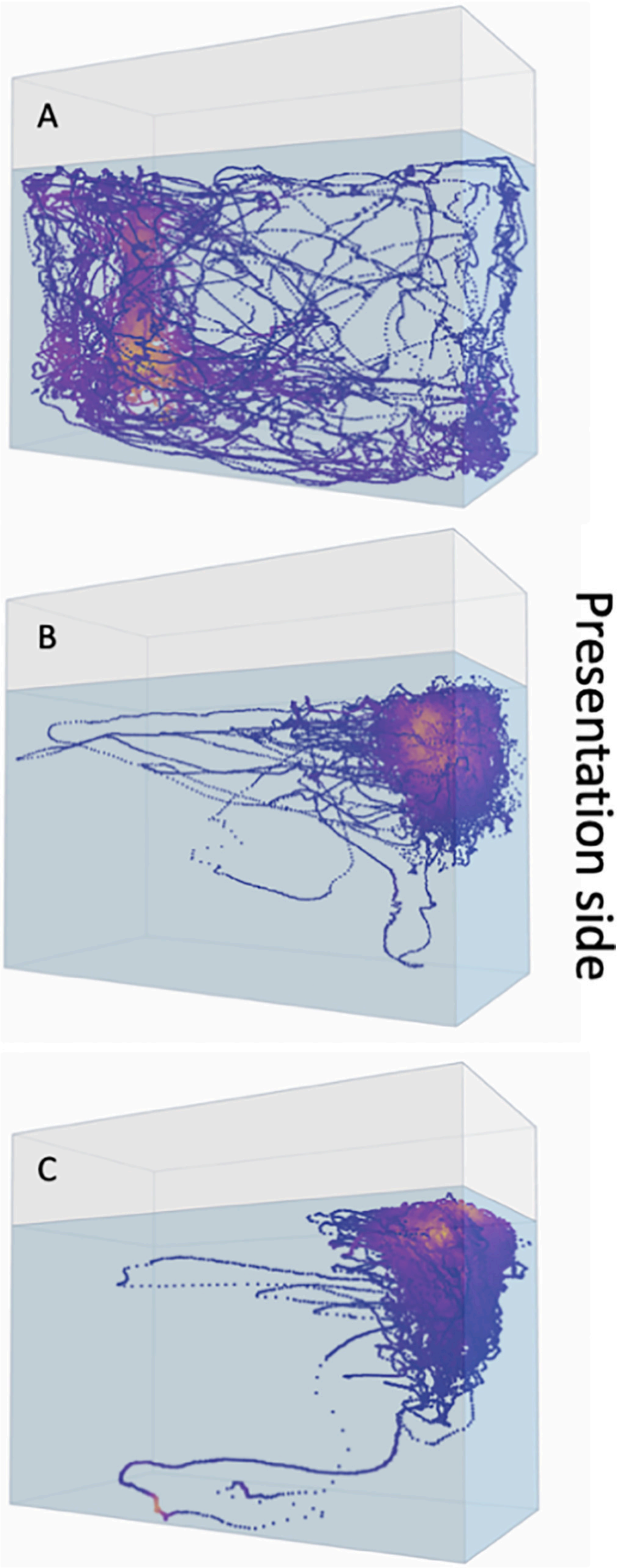

Fig. 2. Representative 3D tracks of fish presented with (A) an empty tank (control); (B) a conspecific in an adjacent tank and; (C) a mirror.

Table 2
Descriptive statistics, main effects and post-hoc comparisons of variables related with activity and position in the tank of fish presented with an empty tank (control), conspecific in an adjacent tank, and mirror image.

	Control mean ± S.E.	Conspecific mean ± S.E.	Mirror mean ± S.E.	Main effects				Post-hoc comparisons - p		
				F	d.f.	p	ω^2	Control vs conspecific	Control vs mirror	Conspecific vs mirror
Total distance travelled (cm)	1837.71 ± 219.55	3844.59 ± 409.39	3235.72 ± 320.69	9.978	2,30	<0.001	0.352	<0.001	0.010	0.387
Time close to stimuli^{1} (s)	570.63 ± 107.82	1734.21 ± 25.22	1606.20 ± 64.93	83.193	2,30	<0.001	0.833	<0.001	<0.001	0.185
Time close to surface^{1} (s)	487.33 ± 55.62	1713.64 ± 29.91	1461.17 ± 81.49	85.348	2,30	<0.001	0.836	<0.001	<0.001	0.113
Frequency of air breathing^{2}	18.58 ± 2.29	95.36 ± 11.86	91.42 ± 12.41	27.480	2,32	<0.001	0.602	<0.001	<0.001	0.802

F: Anova-test F-estimate; p: p-value; ω^2: effect size estimate (omega squared); statistically significant values are in bold. 1. Variable was squared-transformed for the analysis; 2. Variable was log-transformed for the analysis.

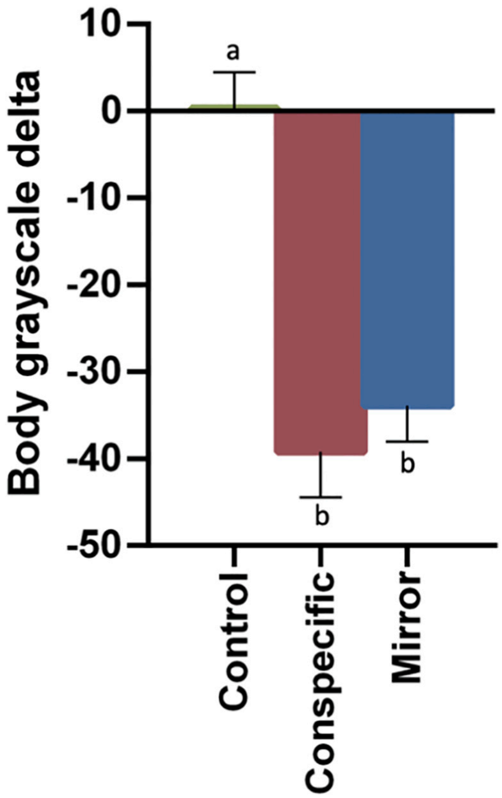

Fig. 3. Change in body grayscale from the acclimation to the test phase in fish from the control (N = 11), conspecific (N = 10) and mirror (N = 12) groups. A body grayscale delta value of 0 represents no change between phases while negative variation indicates a darkening of the body. Values are mean ± S.E. Different letters indicate significant differences between groups at $p < 0.001$.

3.2. Hormones

There was a clear surge in plasma androgen levels after the 30 min aggression challenge. Average levels of KT were, respectively, 3.8 and 6.1 times higher in fish from the conspecific and mirror treatments as compared with the control group ($F_{(2,32)} = 17.341$, $p < 0.001$, Fig. 5). The difference between groups for T levels was also significant, although the increase after the aggression challenge was not as pronounced as for KT, with T levels increasing approximately twofold in the aggression groups ($F_{(2,31)} = 14.962$, $p < 0.001$. Fig. 5). For both androgens, there were no statistically significant differences between the mirror image and the interacting conspecific groups (Fig. 5). Considering only fish from the conspecific and mirror groups, there was no correlation in the levels of the two androgens ($r = 0.237$, $N = 23$, $p = 0.276$). The ratio KT/T, which may provide an indication of the conversion rate of T into KT, also did not differ between groups ($F_{(2,31)} = 0.682$, $P = 0.513$, Fig. 5).

The surge in plasma androgen levels after the aggression challenge may suggest a positive modulation of aggressive displays by these hormones. However, while T levels did not correlate with any of the measured aggressive behaviors ($p > 0.231$), KT levels were unexpectedly negatively correlated with the time spent with open opercula ($r = -0.533$, $N = 23$, $p = 0.009$) and marginally with the time with distended fins ($r = -0.399$, $N = 23$, $p = 0.059$) ($p > 0.155$ for the other variables).

Similarly to androgens, plasma F levels also increased in response to the aggression challenge ($F_{(2,27)} = 6.435$, $p = 0.005$) and similarly in both aggression-eliciting groups (Fig. 5). Considering only fish from the conspecific and mirror groups, F levels did not correlate with androgen levels ($p > 0.350$). Low levels of F have been associated with high aggression while physical activity has been shown to increase the levels of this hormone. However, considering only fish from the mirror and conspecific groups, we found no evidence for an association between F levels and threat ($p > 0.351$) or attack ($p = 0.927$) behaviors nor with the frequency of surface air breathing ($p = 0.220$).

Table 3
Descriptive statistics and main effects of aggression variables of fish presented with a conspecific in an adjacent tank or a mirror image.

	Conspecific		Mirror		Main effects			
	N	Mean ± S.E.	N	Mean ± S.E.	t	d.f.	p	d
Threat behaviors								
Time with open opercula (s)	11	372.07 ± 11.88	12	368.39 ± 12.45	0.063	21	0.951	0.028
Time with fins distended (s)	11	1771.31 ± 2.36	12	1588.44 ± 29.53	1.493	21	0.150	0.638
Frequency of caudal swings	11	49.09 ± 5.97	12	80.33 ± 7.46	−0.946	21	0.355	0.476
Attack behaviors								
Frequency of bites/head hits^{1}	11	95.27 ± 7.21	12	19.83 ± 2.5	2.219	21	**0.038**	0.868

N: sample size; t: Student T-test estimate; d.f.: degrees of freedom; p: p-value; d: effect size estimate (Cohen's d); statistically significant values are in bold. 1. Variable was log-transformed for the analysis.

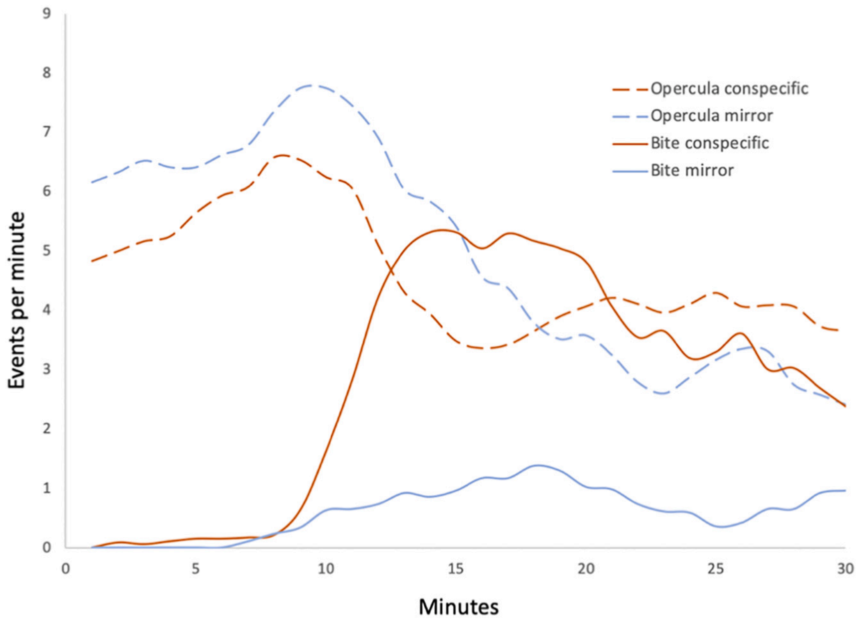

Fig. 4. Temporal variation in the frequency per minute of opercula displays and bites/head hits towards a conspecific in an adjacent tank and a mirror image. Results are displayed as a four-point moving average.

3.3. Mirror vs conspecific

Reduction of data dimension was obtained by a PCA including all variables listed in Tables 2 and 3, body grayscale delta (Fig. 3), and plasma levels of KT, T and F (Fig. 5). The first three factors of the PCA explained 72% of the variance (Supplementary Table 1). Fish from the control group formed a separate cluster but there was significant overlap between the conspecific and mirror animals (Supplementary Fig. 2). An LDA on the scores of the PCA factors correctly classified all control fish in their group (Fig. 6). Contrarily, there was a significant misclassification of fish from the conspecific group into the mirror group (5 out of 12, 41%) while the opposite was less pronounced (2 out of 12, 16%).

4. Discussion

The objectives of this work were to contribute to two still unresolved topics in the study of aggression in fish: (1) the role of androgens and corticosteroids as modulators of agonistic displays and (2) the usefulness of the mirror test as a standardize experimental procedure to measure aggression.

4.1. Androgen levels increase after mirror and conspecific fights

There was a pronounced increase in KT and less pronounced in T plasma levels in fish responding to the aggression challenge, regardless of stimulus type. These results contrast with a previous study in *B. splendens* where KT levels were found not to vary after an interaction with a conspecific behind a partition (Dzieweczynski et al., 2006). However, in that study KT levels were measured from the water and not from plasma and a correlation between water and plasma levels could not be demonstrated. Measuring steroids from water needs to be carefully validated for any new species (Scott et al., 2008). In the Dzieweczynski et al. (2006) study, after interacting for 20 min, males were transferred from the test tanks into containers for 2 h for hormone collection from the water and it is possible that plasma KT levels, or KT secretion, quickly decayed after the end of the interaction, explaining the absence of differences between treatments.

As far as we know, this is now the fourth study reporting androgen responses to unresolved fights in fish and the first in non-cichlids. In males of *A. burtoni*, plasma KT and T levels increased in response either to a mirror or to an opponent behind a partition (Desjardins and Fernald, 2010 but see Oliveira and Canário, 2011). A similar increase in KT and T after a 20 min mirror fight was reported for *Pundamilia* spec. males but a comparison with conspecific fights was not included (Dijkstra et al., 2012). In contrast, in the Mozambique tilapia *Oreochromis mossambicus*, KT and T levels failed to increase in response to mirror images, while increasing in response to territorial intrusions by conspecific males (Hirschenhauser et al., 2004; Oliveira et al., 2005). Thus, of the four studies conducted so far where the androgen response to unsettled fights was measured, three demonstrated an increase in plasma levels. Differences in the results obtained between these studies may relate to species variation in the response to mirror images or to methodological differences (Oliveira and Canário, 2011).

These results should be interpreted in light of several hypothesis that have been put forward to explain the androgen response to aggressive challenges. On one hand, androgens may play a role during the ongoing fight, facilitating the expression of aggressive behaviors. This has been suggested for a few species where administration of androgens increased aggressive displays (Gonçalves et al., 2017; Oliveira and Gonçalves, 2008, 2008; for a meta-analysis see Hirschenhauser and Oliveira, 2006), including for *B. splendens* (Forsatkar et al., 2013). However, there was no positive correlation between the duration or frequency of aggressive displays and androgen levels, similarly to what has been reported in other fish species (e.g. Desjardins et al., 2006). Indeed, the only correlation found between aggressive behavior and androgen levels was in an opposite direction, with post-fight KT levels being negatively correlated with threat displays. It has recently been shown that *O. mossambicus* males have two KT peaks after a territorial intrusion, one at 2–5 min and another at 60–90 min (Félix et al., 2020). In our study, the mutual assessment phase occurred within the first 8–9 min of the fight and it is possible that KT may have responded more in fish with more frequent displays, with compensatory mechanisms bringing its levels further down as compared with less responsive fish by the end of the trial (30 min), when blood was collected. The absence of a positive correlation between post-fight androgen levels and the frequency of aggression displays may also be explained if the magnitude of the change in hormone levels, rather than absolute concentration, is more relevant for the modulation of behaviors. However, further studies investigating the temporal covariation between aggression and androgen levels during *B. splendens* fights are needed to test these hypotheses. Further,

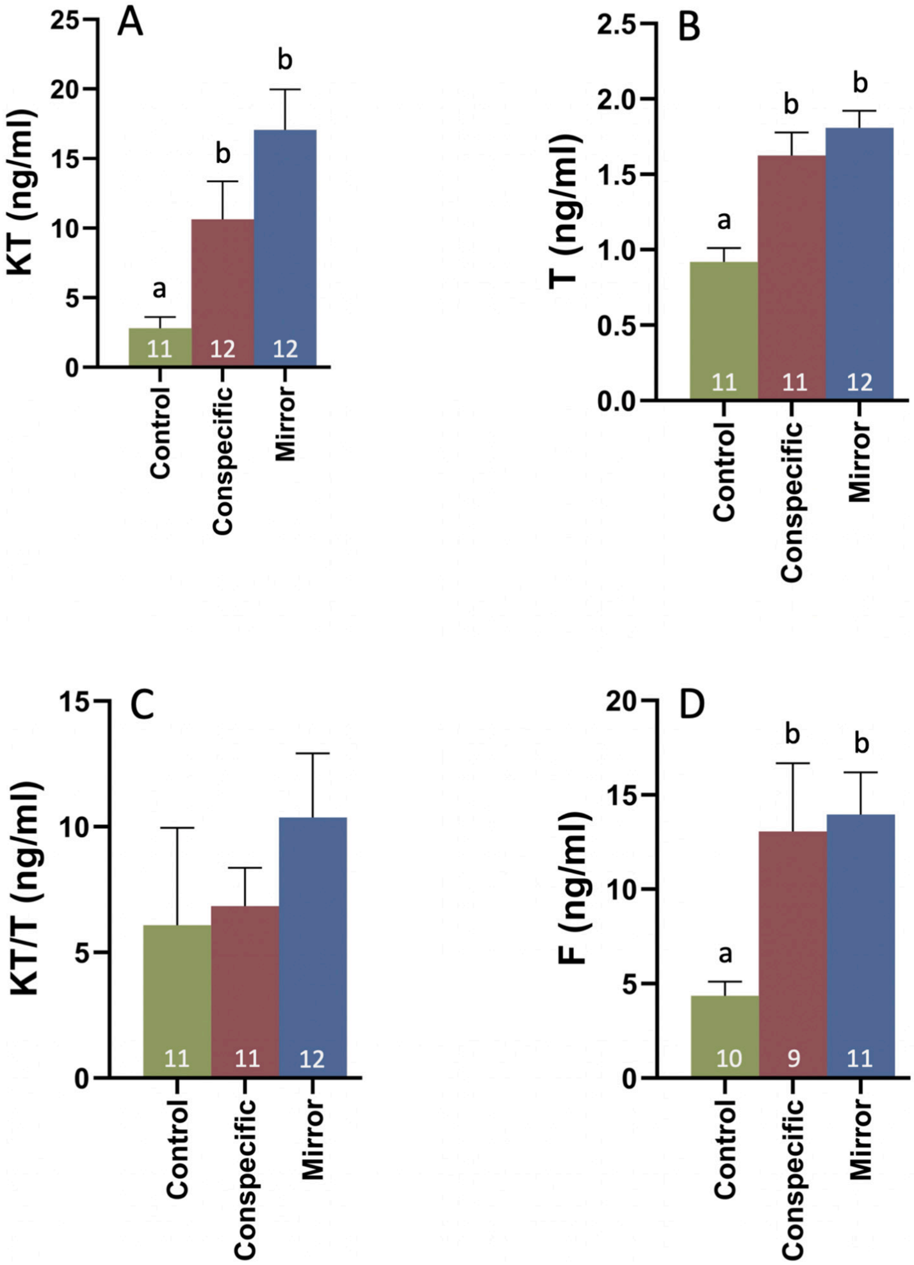

Fig. 5. Plasma levels of (A) 11-ketotestosterone (KT); (B) testosterone (T); (C) ratio KT/T and; (D) cortisol (F) in fish exposed to a control (empty tank), conspecific in an adjacent tank, and mirror image. Values are mean ± S.E. Numbers in columns are sample size. Different letters indicate significant differences between groups at $p < 0.05$.

gonadectomy, which is expected to reduce peripheral androgen levels, did not have an impact in aggression levels in male *B. splendens* (Weiss and Coughlin, 1979), corroborating results found for other fish species (Almeida et al., 2014; Oliveira and Gonçalves, 2008), and further questioning the role of androgens as facilitators of aggression in this species. Second, the androgen response may depend on the outcome of the fight (winning/losing), modulating the behavioral response in future interactions, as suggested under the challenge hypothesis (Wingfield et al., 1990). The absence of androgen response to mirror images in *O. mossambicus* was seen as supporting this hypothesis (Hirschenhauser et al., 2004; Oliveira et al., 2005, see also Oliveira et al., 2009). However, in our study fighting a mirror image or a conspecific behind a partition was unlikely to trigger a loser/winning experience and indeed no submissive displays were recorded in any of the fish. Future modulation of aggression based on previous outcome is thus unlikely to explain the observed androgen surge after the 30 min unresolved fight. Third, a study on sockeye salmon *Oncorhynchus nerka* has shown a KT and T surge within 3 min of exposure to an acute stressor, suggesting that fast androgen secretion can play a role in the stress-response. Nevertheless, in that study, the androgen response was no longer present 30 min after the stressor was applied. Further, while in our results there was an increase in F after both the mirror and conspecific fight, androgen levels did not correlate with F, suggesting a separate mechanism for the activation of androgens and corticosteroids peripheral secretion. Finally, circulating androgens may not play a role in the central modulation of aggression but instead act peripherally. In humans, androgens have been shown to increase after acute physical activity and to induce insulin-related effects in skeletal muscles by upregulating glucose transporter type 4 (e.g., Antinozzi et al., 2017). The increase in plasma KT and T may thus be related with modulation of

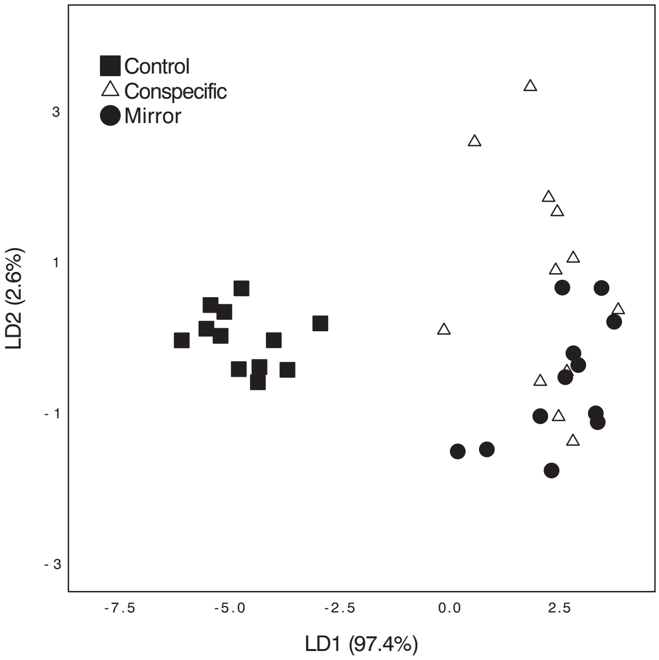

Fig. 6. Clustering of fish used in the experiments based on the measured endocrine and behavioral variables by the two first coefficients (LD1 and LD2) of a linear discriminant analysis. Numbers in the axis's legend represent the percentage of variation explained by each coefficient.

glucose uptake by skeletal muscles in response to the aggression challenge. Although no correlation was found between post-fight androgen levels and activity-related variables, it is still possible that the surge in androgens were related with preparing skeletal muscles for sustained activity and/or recovery (Kraemer et al., 2017). Future experiments testing the effect of androgens in prolonged physical exercise and recovery in *B. splendens* should be able to test this hypothesis.

It should be highlighted that these hypotheses are not necessarily mutually exclusive. For example, the increase in androgen secretion may play a role during the current fight but also prepare the animal for future contests. In fact, in the East African cichlid fish *Pundamilia* spec., fighting a mirror increases androgen levels and also the possibility of winning future fights (Dijkstra et al., 2012) and androgens have been shown to mediate the winner effect (Oliveira et al., 2009). Thus, the aggression challenge may signal an increase in the instability of the social environment, triggering androgen secretion that may play a role both during present and future fights. As far as we know, androgen levels between winners and fish from unsettled fights have not been directly compared and it is not clear if winner effects may be mediated by longer lasting or more acute androgen changes in response to resolved fights. However, males of *B. splendens* are highly aggressive and it is difficult to design a sound winner/loser experiment that complies with ethical procedures as males may get seriously injured or even die during fights.

It has recently been shown for *O. mossambicus* that the androgen response to a challenge was variable and dependent on previous baseline levels of androgens, where only fish with low baseline levels were able to mount a response (Félix et al., 2020). Although we did not collect baseline endocrine data, there was low overlap in KT and T levels between the mirror/conspecific and control animals, suggesting that either this mechanism does not occur in *B. splendens* or that most tested fish had low baseline androgens levels, being able to mount a response. Further, while Félix et al. (2020) suggest a population wise absence in KT response to the challenge, as this androgen increased in some fish but decreased in others after the interaction, in our study there was a clear overall increase in KT levels in fish exposed to a fight. Thus, it seems that the androgen response to aggressive interactions may vary both between and within species calling for further investigation of this topic across phylogenetic groups and taking into consideration context and individual variation.

Taken together, the results show a clear and pronounced surge in androgen secretion after unresolved fights. However, the functional role of the post-fight androgen increase in *B. splendens*, and in particular the putative relationship with either current or future aggressive behavior, remains to be clarified, not only for this species but for vertebrates in general.

4.2. Cortisol levels increase after mirror and conspecific fights

This is the first study in fish showing an increase in F levels after mirror fights or after any other type of unsettled fight. This hormone has been classically linked to the stress response but evidence has been accumulating associating F to aggressive behavior in fish (Gonçalves et al., 2017). As for androgens, the results for F may have different explanations. One possibility is that F facilitates the expression of aggressive behavior. For example, in the dusky gregories *Stegastes nigricans*, territorial intrusions by both conspecific and heterospecific fish caused an increase in F (but not in androgens) that correlated highly with aggressive behavior (Ros et al., 2014). However, opposite results have been found in other fish species. In smallmouth bass (*Micropterus dolomieu*) administration of F did not impact aggressive behavior displayed in the context of nest defense, while an androgen receptor antagonist had a negative impact (Dey et al., 2010), and simulated nest intrusions increased T but not F levels (O'Connor et al., 2011). Also, in trout (*Oncorhynchus mykiss*) lines selected for low and high F responsiveness, the pattern was opposite with fish selected for low-F attaining more frequently a dominant status as compared with fish selected for high-F (Pottinger and Carrick, 2001) and with low F responsiveness animals being more aggressive (Øverli et al., 2004). In this same species, short-term exposure to F did not have an effect on aggression while long-term treatment inhibited these displays (Øverli et al., 2002), highlighting a complex relationship between F and aggression. In our study, post-challenge F levels did not correlate with the expression of aggressive behaviors, questioning the link between F and aggression. Alternatively, the increase in F could represent a stress response. In *O. mossambicus*, a 3 min territorial intrusion caused a rapid (within 2 min) increase in F levels, no longer present after 30 min (Félix et al., 2020), which was interpreted as the intruder being appraised as an acute stressor. Here, the aggressive interaction was much more prolonged, 30 min, probably justifying the three-fold difference in F levels between fish from the aggression and control groups. Although, no correlation between F and activity variables was detected, as found in some stress studies (e.g., Øverli et al., 2004), it is still possible that these results reflect a general activation of the hypothalamus-pituitary-interrenal axis at the beginning of agonistic interactions. The similar post-fight F levels in fish exposed to the mirror image or conspecific further suggest that this activation is independent of stimuli type, questioning the interpretation by Desjardins and Fernald (2010) that mirror images, but not interactions with a live conspecific, elicit a fear response.

Future experiments investigating the link between F and aggression in *B. splendens*, in particular through the manipulation of F levels, should be able to clarify the role of this hormone in the modulation of agonistic displays.

4.3. Similar but not identical responses to a mirror and a live opponent

The response to the mirror image and live opponent was generally similar. There was a marked increase in the frequency of air breathing, a measure of metabolic effort (Alton et al., 2013), when fish were displaying aggression and this increase was similar in response to the mirror image and conspecific. This variable correlated with total distance travelled and time distending fins and these measures did not differ between the mirror and conspecific trials, justifying the comparable frequency of air intake. The frequency of attacks did not have an impact on overall metabolic effort, closely matching the results reported for this species by Castro et al. (2006). A previous study for a wild-type and a fighter strain of *B. splendens*, and for both males and females, had already suggested an equivalent metabolic effort in response to mirror images and interacting live conspecifics (Ramos and Gonçalves, 2019). On the contrary, Arnott et al. (2016) found that mirror fights elicited more frequent air breathing than an interaction with a conspecific behind a partition in *B. splendens*. As in that study the frequency or intensity of aggressive behaviors did not differ between treatments, the authors interpreted the results as possibly arising from a lower motivation to display to the mirror, allowing the aggressive displays to be interrupted more frequently by subordinate activities. Differences in the results here presented to the Arnott et al. (2016) study are not clear but may relate to the used strains or to methodological disparities (e.g., in the Arnott et al. (2016) study an undefined commercial strain was used, and trials had a duration of only 15 min).

The duration and frequency of all measured threat displays were similar for the mirror and conspecific trials. These results parallel another study in *B. splendens* where a mirror image or a live conspecific used as a social reinforcer held comparable results in the frequency of threat displays (opening of the opercula; Eisenreich et al., 2017). Similar results were found by Ramos and Gonçalves (2019) in the study comparing the aggressive behavior of a fighter and a wild-type strain. Unlike threat behaviors, overt aggression, as measured by the frequency of attempted bites/head hits, was higher towards conspecifics than towards the mirror image. During paired-staged fights, male *B. splendens* switch from a mutual assessment phase that starts within the first seconds of the fight to overt aggression after 2–6 min, without much variation, and fights can last several hours before being decided (personal observations from the authors conducted in *B. splendens* fighting rings in Southeast Asia, see also Braddock and Braddock, 1955; Simpson, 1968). After the overt aggression phase begins, the pair alternates between episodes of surface-air breathing, biting/striking and mouth-locks (see also Vu et al., 2020). This suggests that mirror images provide the necessary cues for fish to transition from the mutual assessment to the overt attack phase and, because there were no significant differences in the latency of the first bite between stimuli type, that this is not dependent on asymmetric elements of the fight. On the other hand, after transitioning to the overt aggression phase, differences between the mirror and conspecific stimuli lead to more frequent direct attacks towards the latter. Mirror fights differ from live fights in a number of ways. First, only symmetrical fight elements are reproducible by mirror images. Anti-parallel postures (head-tail) or any other non-symmetrical postures (e.g., head-down submissive posture of one fish) are not possible in the mirror test. Second, although both types of stimuli are interactive, feedback received from the mirror is phased-locked with the behavior of the focal fish while the live opponent feedback is not. Non-reversing mirrors allow head-tail positions but not other non-symmetrical postures nor receiving feedback that is different from the behavior of the focal fish. Still, this technique has been shown to increase the aggressive response in the mangrove rivulus fish, *Kryptolebias marmoratus* (Li et al., 2018a) and in the chub *Squalius cephalus* (Hubená et al., 2020) and could be further explored with *B. splendens* to study the relevance of anti-parallel postures for triggering attacks.

Taken together, the results show that aggressive behaviors elicited by a mirror image did not differ from those displayed towards a live conspecific behind a partition during the mutual assessment phase but that overt aggression was more intense towards a live conspecific. This suggests that asymmetrical or out of phase elements of the interaction are relevant for *B. splendens* males to escalate fights but not during the initial phase of the contest. It should also be highlighted that during the overt aggression phase the mirror image elicited the same behavioral repertoire as the live stimuli, with differences being only quantitative. Together with the similar metabolic and endocrine response to the two stimuli types, this supports the use of mirrors as appropriate aggression-eliciting stimuli in *B. splendens* although caution should be taken whenever overt aggressive behaviors are being studied.

Differences in behavioral output are expected to be paralleled by physiological and neurobiological changes. For example, the aggressive response of *K. marmoratus* to regular mirrors was less pronounced than towards a live opponent and this difference was reflected in the pattern of expression of the immediate early-genes (IEGs) *egr-1* and *c-fos* in brain macroareas (Li et al., 2018b). In the same study, the behavioral response to non-reversing mirrors and an opponent was similar, and accordingly no difference in the pattern of expression of IEGs was detected. However, it has been argued that even in species where mirrors elicit a comparable behavioral output and endocrine response, the cognitive perception of the threat may differ to that of a live opponent. For example, in the zebrafish *Danio rerio* males winning fights displayed a similar amount of aggressive behavior as males fighting their mirror image but the brain transcriptome profiles of winners and mirror fighters differed (Oliveira et al., 2016). Also, in the cichlid *Astotilapia burtoni* the aggressive and endocrine (KT and T) responses to a mirror or to an opponent behind a partition were comparable but expression of *egr-1* and *c-fos* in brain areas homologous to the amygdala and hippocampus was higher towards the mirror, which was interpreted as the mirror eliciting a fear response (Desjardins and Fernald, 2010). Still, this would suggest that, in spite of differences in cognitive perception or brain areas activation, aggressive output would be similar, validating the mirror test as appropriate to measure aggressive behavior. In our study, the higher frequency of attacks towards the live opponent than towards the mirror was not reflected in the endocrine response, as measured by post-fight androgen and corticosteroid plasma levels, which were similarly elevated in both groups. This suggests that putative differences in central processes underlying the higher rate of attacks towards conspecifics in *B. splendens* are unrelated with peripheral levels of androgens and corticosteroids.

A PCA including all behavioral and endocrine variables failed to discriminate fish from the mirror and conspecific group (Supplementary Fig. 2) while an LDA wrongly classified 29% of the subjects. Taken together, the results show a very similar aggressive and endocrine response towards the mirror image and conspecific.

In conclusion, the choice of the appropriate test to measure aggression in fish in laboratory settings will depend on the objectives of the study. Investigating questions related with resource-holding potential or winner-loser effects is likely to only be possible in species where real interactions are feasible and ethically acceptable. On the other hand, for studies that only aim to elicit a significant aggressive response from the study subjects, for example to investigate behavioral or physiological aspects of aggression, the mirror test or the use of live conspecifics behind partitions might be adequate in species that respond meaningfully to these types of stimuli. If the purpose is to research intrinsic motivation for aggression independently of the dynamics of the interaction, standardize non-interactive stimuli are more appropriate and the use of models, video playbacks or opponents behind one-way mirrors can provide a solution. Nevertheless, the type of test to be used needs to be properly validated as significant variation in the response to these different types of stimuli across species and context has been reported.

The results also show that there is a clear surge in both androgens and corticosteroids during the course of an aggression challenge, independently of stimulus type and before the fight is decided, but the function of this increase remains to be elucidated.

Declaration of competing interest

None.

Acknowledgements

This work was supported by the Macao Science and Technology Development Fund, project 093/2017/A2. We thank two anonymous reviewers and the Associate Editor for their insightful comments on a previous version of the manuscript.

Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.yhbeh.2021.104991.

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