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Original Article

Behavioral and endocrine responses to noninteractive live and video conspecifics in males of the Siamese fighting fish

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Abstract

The physiological mechanisms underlying variation in aggression in fish remain poorly understood. One possibly confounding variable is the lack of standardization in the type of stimuli used to elicit aggression. The presentation of controlled stimuli in videos, a.k.a. video playback, can provide better control of the fight components. However, this technique has produced conflicting results in animal behavior studies and needs to be carefully validated. For this, a similar response to the video and an equivalent live stimulus needs to be demonstrated. Further, different physiological responses may be triggered by live and video stimuli, and it is important to demonstrate that video images elicit appropriate physiological reactions. Here, the behavioral and endocrine responses of male Siamese fighting fish *Betta splendens* to a matched-for-size conspecific fighting behind a one-way mirror, presented live or through video playback, were compared. The video playback and live stimulus elicited a strong and similar aggressive response by the focal fish, with a fight structure that started with stereotypical threat displays and progressed to overt attacks. Postfight plasma levels of the androgen 11-ketotestosterone were elevated as compared to controls, regardless of the type of stimuli. Cortisol also increased in response to the video images, as previously described for live fights in this species. These results show that the interactive component of a fight and its resolution are not needed to trigger an endocrine response to aggression in this species. The study also demonstrates for the first time in a fish a robust endocrine response to video stimuli and supports the use of this technique for researching aggressive behavior in *B. splendens*.

Key words: aggression, androgens, corticosteroids, *Betta splendens*, one-way mirror, video playback.

Hormones are thought to explain a significant part of the variation in aggression levels both within and across species (Trainor and Nelson 2012). In particular, androgens of gonadal origin have been proposed to explain why males are generally more aggressive than females (Edwards 1969) and dominants more aggressive than subordinates (Taves et al. 2009). However, several experimental studies in fish have failed to confirm this hypothesis. For example, removing gonadal androgen input through gonadectomy does not suppress aggression levels in some fish species (Weiss and Coughlin 1979; Almeida et al. 2014), and exogenous administration of androgens does not always lead to an increase in aggression (e.g., Kindler et al. 1991). This contradictory evidence has prompted the search for other possible physiological modulators of aggression in fish. For example, nonapeptides such as isotocin (IT) and arginine vasotocin (AVT), the fish equivalents to oxytocin and vasopressin in mammals, respectively, have been associated with aggression in fish, although with variable results. AVT, but not IT-related genes, is overexpressed in the brain of dominant males in the zebra fish *Danio rerio* (Filby et al. 2010) and accordingly, agonistic interactions seem to be more associated with changes in brain levels of AVT than with IT (Teles et al. 2016). In the 3-spined stickleback *Gasterosteus aculeatus*, mRNA levels of both AVT and IT are associated with male aggression, although at different stages of the reproductive cycle (Kleszczyńska et al. 2012). However, exogenous administration of these nonapeptides, or of specific antagonists, in fish has confirmed a positive association with aggression in some cases (e.g., Semsar et al. 2001; Santangelo and Bass 2010; Oldfield and Hofmann 2011) but not in others (e.g., Bastian et al. 2001; Semsar et al. 2001; Lema and Nevitt 2004). Likewise, other steroid hormones, in particular corticosteroids, have been identified as possible modulators of aggression. In general, it has been shown that individuals with low basal levels of cortisol (F) are more aggressive (Sloman et al. 2001) and that exogenous F administration reduces aggression (Gilmour et al. 2005), suggesting an inverse relationship between corticosteroids and aggressive behavior. However, similarly to androgens and AVT and IT, other studies in fish have shown that plasma F levels increase after unresolved fights (Félix et al. 2020; Ramos et al. 2021) and corticosteroid levels have been found to correlate positively with aggressive behavior (Ros et al. 2014), also questioning the role of these hormones in the modulation of aggression.

These contradictory results may partially be a consequence of a lack of standardization in experimental procedures between studies. For instance, different types of aggression-eliciting stimuli have been used in studies investigating aggressive behavior in fish (for a review in zebra fish, see

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Way et al. 2015). These include the use of paired fights with fish placed in the same arena (e.g., Vu et al. 2020), stimuli presented behind a transparent partition (e.g., Ramos and Gonçalves 2019), mirror (e.g., Meliska et al. 1980) and nonreversed (e.g., Li et al. 2018) mirror images, stimuli presented behind a one-way mirror (e.g., Meliska et al. 1980), static 2D or 3D models (e.g., Halperin et al. 1992), robots (e.g., Romano et al. 2017), or video playback, that is, the presentation of stimuli in video (e.g., Allen and Nicoletto 1997). Some of these stimuli are interactive and asymmetric as feedback received varies according to the behavior displayed by the focal fish, as is the case of opponents in the same arena or behind transparent partitions, interactive video playback, and interactive robots. Others are interactive but symmetric as the feedback received is equivalent to the behavior displayed by the focal animal, such as mirrors and reversed mirrors. One-way mirrors, static models, noninteractive video playback, and robots provide a stimulus that is invariant to the focal fish response. The choice of the type of test to be used depends on the characteristics of the species under study and the objectives of the work. For example, while live fights, where opponents are placed in the same arena, allow a resolution of the challenge with a winner and a loser being determined, this is more difficult or impossible to achieve with other types of tests. However, it may not be ethical for highly aggressive species to conduct live fights as animals can get injured or even die. If what is intended is comparing aggression levels across experimental groups, it may be more adequate to present animals with standardized stimuli that do not vary with the behavior of the focal animal and that are the same across trials. 2D and 3D models have been used for this purpose, but they generally lack motion and may not be perceived as meaningful stimuli (Way et al. 2015). A few studies in fish have used robots, and this is a promising technique as it allows a 3D interaction with a performing stimulus (Romano et al. 2017). However, to build a robot that is recognized as a conspecific is complex, and many labs lack the human resources and equipment to implement this technique.

If properly validated, video playback provides a powerful tool for the study of behavior. This technique allows the presentation of the same invariant stimulus to animals from different treatments or along different periods of time, minimizing variation across trials. Further, digitization and animation techniques allow precise control that can be used to produce synthetic animations to test the response to particular features (e.g., Gonçalves et al. 2000; Fisher and Rosenthal 2006), or to design interactive video playback experiments where the stimulus output depends on the behavior of the focal animal (e.g., Butkowski et al. 2011). Video playback has been used for several decades in fish studies but with variable levels of success. While some species respond meaningfully to video playback, others do not seem to recognize images in videos or present only a weak response, and this variation is thought to be related to sensorial differences across species (for a discussion, see Chouinard-Thuly et al. 2017; Oliveira et al. 2000; and other articles in that volume). A commonality to the use of artificial stimuli to study behavior is thus the need to properly validate that they are being perceived meaningfully. For this, equivalent artificial and natural stimuli should be used for comparison, which is not always the case. For example, all studies in fish validating noninteractive video playback compare it with interactive stimuli, not allowing to disentangle the artificial/live component from the interactive/noninteractive component (e.g., Balshine-Earn and Lotem 1998). Also, several studies only compare time spent in association with live and video stimuli as a validation measure of video playback (e.g., Clotfelter et al. 2006; Gómez-Laplaza and Gerlai 2021) without controlling for possible cues in video (e.g., motion) that can attract/repel the focal animals, making it difficult to understand if video images are indeed being perceived meaningfully. Further, while behavioral output is usually measured, no study so far has used physiological endpoints to compare the response to video playback and live stimuli in fish. This is relevant because it has been suggested that the physiological response to different stimuli may differ, even when the behavioral output does not (Desjardins and Fernald 2010).

The Siamese fighting fish *Betta splendens* offers an excellent model to test the suitability of video playback as a tool to study aggression and, more generally, the role of hormones as modulators of aggressive behavior. First, its aggressive behavior has been well characterized (Braddock and Braddock 1955; Simpson 1968) and it consists of stereotyped threat and attack displays than can be quantified without bias. Second, video playback has been previously used with apparent success, suggesting that the species perceives video images and animations meaningfully (Allen and Nicoletto 1997; Clotfelter et al. 2006; Verbeek et al. 2007; Dzieweczynski et al. 2014; Dzieweczynski and LaMonica 2016; Dzieweczynski and Kane 2017; Neri 2019). Third, the physiological response to an aggressive interaction in males of this species includes a robust increase in plasma levels of androgens and corticosteroids (Ramos et al. 2021; Ramos and Gonçalves 2022), and these can be used as a physiological indicator of the validity of video playbacks.

Here, we compared the behavioral and endocrine responses of male *B. splendens* to video playback and one-way mirror fights. For the one-way mirror test, a conspecific was observed by the focal fish fighting behind the one-way mirror. This provided a noninteractive fight as the conspecific could not observe the focal fish but rather its own image in the reflective side of the mirror. For the video playback trials, conspecific males fighting the one-way mirror were filmed from the same perspective of the focal fish and played back, providing an equivalent stimulus to the live opponent. Aggressive behavior and postfight androgen (11-ketotestosterone, KT) and corticosteroid (cortisol, F) plasma levels were measured. Overall, the study aimed to test and validate the use of video playback to investigate the endocrine regulation of aggressive behavior in *B. splendens* and to provide additional data on postfight endocrine responses to aggression in this species.

Materials and Methods

Animals

Siamese fighting fish used in this experiment was 17 months old from the F1 generation of a cross between a wild type and a fighter strain raised in mixed-sex groups (see Ramos et al., 2021, for more details on this line). Thirty-six males were isolated at 10 months old into 9W × 9D × 20 H cm tanks, containing a small ceramic shelter, with no visual contact with other conspecifics. Fish were fed twice a day with pellets and live artemia, except on the day of the experiment where they were not fed, and maintained under a 12L:12D photoperiod and water temperature of 28 ± 1 °C. Reverse osmosis water for stock and isolation tanks was conditioned with Indian almond tree leaves and salinity kept at 250 ppm. Two months prior to the beginning of the experiment, fish were transferred

to new individual tanks of the same dimension as the experimental tanks (25 W × 12.5 D × 20 H cm). Fish were netted and released back into their tank daily for 1 week before the experiment to habituate to handling.

Experimental procedure

A behavioral paradigm was used to study aggressive behavior without the influence of interaction between the focal and stimulus fish. Fish were randomly assigned to 1 of 4 treatments: noninteractive one-way mirror conspecific (n = 8), noninteractive one-way mirror conspecific control (n = 7), noninteractive video playback (n = 10), and noninteractive video playback control (n = 7). Between groups, fish did not differ in weight (W) or standard length (SL) (one-way ANOVA, W, $F_{3,32} = 1.006$, $P = 0.403$; SL, $F_{3,32} = 1.118$, $P = 0.356$). Fish used as stimuli, both live and in video playback, and as focal were matched for size (SL, C.V. <10%). The experiment was performed in an arena with 122 W × 57 D × 57 H cm, composed of white walls and 2 white sliding doors. A diffuse LED panel provided general illumination to the arena.

For the one-way mirror setup, the tank of the focal fish was separated from the tank of the stimulus conspecific by a 1-cm-thick one-way mirror (Figure 1A). Two opaque smart screens that become transparent when activated prevented the focal fish from seeing the stimulus tank side and the stimulus fish from seeing the mirror during the acclimation period. The tank of the stimulus conspecific was narrower than the focal fish tank to avoid large variation in target size, with dimensions of 12.5 W × 8 D × 20 H cm. This tank had directly above it a diffuse LED strip to create the light contrast needed for the one-way mirror to be reflective for the stimulus but not for the focal fish. The behavior of the focal fish was recorded using 1 side and 1 top Raspberry Pi camera module V2, and of the stimuli conspecific fish using a similar side camera, all at a resolution of 1,640 × 922 px at 30 fps. Each camera was connected to an independent Raspberry Pi board 4B, with 1 raspberry also controlling the activation of the smart screen via a relay switch. Stimuli fish were used only once. For the one-way mirror control, no fish was added as a stimulus and the focal fish observed an empty tank. Control trials were run with only the focal or stimulus fish added to the setup, ensuring that the focal fish was not viewing its mirror image and that the stimulus fish was responding to its mirror image and not to the focal fish. In the video-playback setup, conditions were similar but an LCD screen (10.1″TFT LCD with LED backlight, refresh rate 60 Hz, 2K, and 2,560 × 1,600 IPS pixels resolution) controlled remotely using a Raspberry Pi board 4B was placed adjacent to the focal fish tank (Figure 1B).

Recording of the stimuli to be played back was done with a Logitech HD Pro Webcam C920 placed inside a dark chamber facing the one-way mirror and stimuli conspecific tank, separated by a smart screen, at a resolution of 1,920 × 1,080 px and frame rate of 30 fps (Figure 1C; Supplementary Video 1). The stimulus fish was placed inside the tank and left to acclimate for 15 min. At the end of this period, the smart screen and video camera were activated, with recordings lasting 30 min. This allowed obtaining 30-min videos of a fish fighting from the opponent's perspective. The size of the fish on the screen was 1:1 when the fish was displayed close to the mirror. A total of 7 fish were recorded and because of the need to match the stimulus and focal fish for size, 1 video was presented to 3 focal, another to 2 focal, and the remaining were only used once. For the video playback control treatment, footage of the tank without any fish was obtained under the same conditions. The brightness of the LCD screen was visually adjusted to match the tank light conditions as seen through the one-way mirror.

To begin a trial, fish were transferred from their housing tanks to the test tank, the cameras were activated, and a 30-min acclimation period was given with the smart screens opaque (one-way mirror trials) or a white screen on the LCD (video playback trials). At minute 30, the smart screens or the test video in the LCD screen were automatically started allowing the fish to see either a live conspecific fighting its mirror image, an empty tank, a video of a conspecific fighting its mirror image, or a video of an empty tank. Observations had a total duration of 30 min. At the end of the observation, the focal fish was immediately removed from the tank, anesthetized with cold buffered MS222 (concentration 600 mg/L), and blood was extracted from the caudal vein using a heparinized 27G syringe. Time for blood extraction since the end of the trial was not recorded but in other similar experiments in our lab (Ramos et al. 2021; Ramos and Gonçalves 2022) blood is collected within 2–5 min. After the procedure, individuals were placed in individual recovering tanks with aeration. Blood samples were centrifuged for 15 min, and plasma was transferred to new tubes and stored at –20 °C until

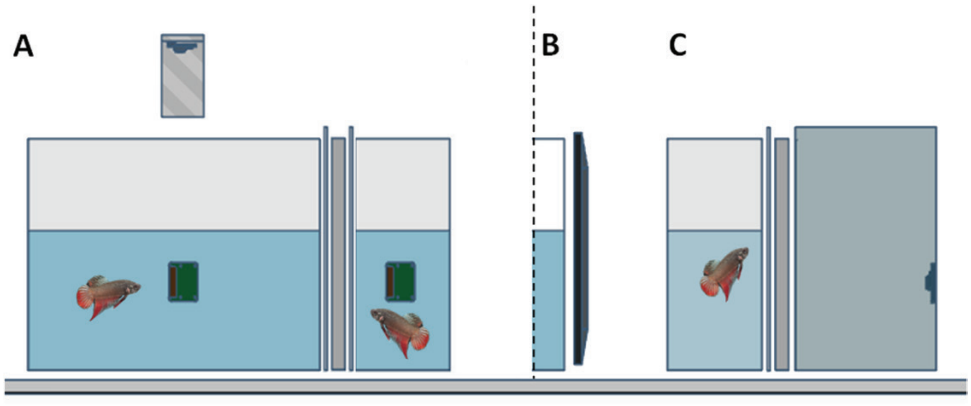

Figure 1. Experimental setup used to test (A) the response to a live conspecific fighting its mirror image or (B) to a video playback of an equivalent stimulus; (C) footage for the video playback was obtained by filming behind a one-way mirror a fish fighting its image. An opaque smart screen that becomes transparent when activated was placed between the one-way mirror and both the focal and the live stimulus tank and turned on after the acclimation period. Trials were recorded with top and side cameras for behavioral analysis.

further analysis. Water in the test tank was changed between experimental trials.

Behavioral analysis

Aggressive behaviors of both the focal and stimulus fish were manually scored with Boris software v.7.9.19 (Friard and Gamba 2016; http://www.boris.unito.it/) and included: duration of open opercular displays, duration of distended fins, duration of darkened skin color, frequency of caudal swings, frequency of charges, frequency of bites and frequency of air breathing. The total distance moved, duration of time spent near the stimuli (within 5 cm), and duration of time spent near the surface of the tank (within 4 cm) were measured using Ethovision XT.

Hormone analysis

Plasma levels of KT and F were measured with competitive enzyme-linked immunosorbent assay (ELISA) kits from Cayman Chemical following the manufacturer's instructions. A lack of interference in the assay of other immunoreactive molecules for this species and for these ELISA kits had already been 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 in a total volume of 50 µL. Thus, plasma volumes of 0.33 and 2.5 µL were used for KT and F, respectively. Experimental samples were measured in the same assay, and the intra-assay coefficient of variation, calculated from the sample duplicates, was 2.86% for KT and 3.20% for F.

Statistical analysis

Parametric procedures were used for all comparisons, with normality and homoscedasticity of data being tested a priori with Shapiro–Wilk's and Levene's tests, respectively. As indicated, some variables were log-transformed to comply with parametric assumptions or nonparametric tests were used when assumptions were still violated after data transformation. First, differences between the 2 control groups in total distance traveled, frequency of air breathing, time spent close to the surface, time spent close to the stimulus tank/screen side, and hormone levels (KT and F) were analyzed with unpaired *t*-tests. There were no differences for any of the variables between these 2 groups ($P > 0.212$), and they were merged for further analysis. For these same variables, differences between the experimental and the merged control groups were tested with one-way ANOVAs with factor treatment (one-way mirror, video playback, control), followed by post hoc Tukey tests to assess differences between groups. Differences in aggressive behavior between the one-way mirror and video playback groups were tested with unpaired *t*-tests or with Mann–Whitney *U*-tests as aggression was not displayed during control trials. All correlations between variables presented were assessed with Pearson's correlation tests.

A principal component analysis (PCA) that included all measured behavioral and endocrine variables was applied to investigate the distribution of fish from each treatment. Variables were standardized prior to the analysis. It was predicted that control fish should form 1 cluster and that fish exposed to the conspecific, live or through video playback, should form another cluster if video playback is equivalent to a live stimulus, or 2 further clusters otherwise. The average distance to the centroid in the first 2 PCA factors was calculated with package "vegan 2.5-7" for R.

All statistical analyses were run with R 4.1.0 (R Core Team 2020).

Results

Behavior

Focal fish exhibited a clear aggressive response regardless of stimulus type (Supplementary Video 1). Activity- and position-related variables and metabolic effort, as determined from the frequency of air breathing (Alton et al. 2013), did not differ between these 2 treatments (Table 1). Likewise, the frequency or duration of aggressive displays and the time spent with aggressive coloration were similar (Table 2).

Interactive live and mirror fights in this species follow a highly stereotyped sequence, starting with threat displays (opening of opercula, distension of fins) and switching to overt aggression (charges, bites) after a few minutes (Vu et al. 2020; Ramos et al. 2021). We investigated whether this also occurred in noninteractive fights and if it could be influenced by the type of stimuli. A two-way repeated-measures ANOVA on the time with open opercula, a threat behavior, showed that it was higher in the first half, regardless of stimulus type (within-effects combat phase, $F_{1,16} = 19.782$, $P < 0.001$; between-effects stimulus type, $F_{1,16} = 0.036$, $P = 0.852$; effects interaction, $F_{1,16} = 0.150$, $P = 0.704$; Figure 2). Attack behaviors were more frequent in the second half of the fight, and again no effect of stimulus type was recorded (bites, within-effects combat phase, $F_{1,16} = 5.960$, $P = 0.027$; between-effects stimulus type, $F_{1,16} = 0.001$, $P = 0.975$; effects interaction, $F_{1,16} = 0.590$, $P = 0.454$; Figure 2). Air breathing, an indicator of metabolic activity (Alton et al. 2013), was higher in the second half of the test, independently of stimulus type (within-effects combat phase, $F_{1,16} = 9.991$, $P = 0.006$; between-effects stimulus type, $F_{1,16} = 0.726$, $P = 0.407$; effects interaction, $F_{1,16} = 2.259$, $P = 0.152$; Figure 2). Accordingly, the frequency of air breathing correlated with the frequency of attack behaviors (bites, $r = 0.537$, $N = 18$, $P = 0.019$) but not with the duration of threat displays (opening of opercula, $r = 0.240$, $N = 18$, $P = 0.338$).

Hormones

There was a marked increase in plasma KT levels after the 30-min aggression challenge. The one-way mirror and video playback aggression stimuli resulted in an average KT increase of 4.2- and 5-fold, respectively, as compared to control (one-way ANOVA on KT log-transformed values, $F_{2,28} = 10.189$, $P < 0.001$; Figure 3). Post hoc comparisons confirmed a significant difference of both the one-way mirror ($P = 0.003$) and video playback ($P = 0.002$) groups to the control group, while there was no difference between fish from the 2 aggression treatments ($P = 0.999$). Postfight KT levels of fish from the aggression-elicited groups did not correlate with aggression or activity variables displayed during fights ($P > 0.095$).

The F response to the aggression challenge was not as robust as for KT, but still, postfight F levels differed between fish from different treatments ($F_{2,29} = 4.721$, $P = 0.017$; Figure 3). When presented with video images of a conspecific, F levels increased almost 3-fold as compared to controls ($P = 0.017$). This increase was less pronounced in fish presented with the live conspecific, with post hoc comparisons with the control ($P = 0.943$) and video playback group ($P = 0.077$) not

Table 1. 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 or white screen (control), an opponent behind a one-way mirror in an adjacent tank (one-way mirror), and video playback of an opponent fighting a one-way mirror (video playback)

	Control, Mean ± *SE*	One-way mirror, Mean ± *SE*	Video playback, Mean ± *SE*	One-way ANOVA main effects			Post hoc comparisons—*P*		
				F	*df*	*P*	Control vs. one-way mirror	Control vs. video playback	One-way mirror vs. video playback
Total distance traveled (cm)[a]	1533.61 ± 123.01	2100.16 ± 130.06	2588.70 ± 379.39	5.444	2, 29	**0.010**	0.092	**0.011**	0.765
Time close to stimuli (s)[a]	687.00 ± 42.41	1474.59 ± 133.57	1181.83 ± 129.84	18.464	2, 29	**< 0.001**	**< 0.001**	**< 0.001**	0.215
Time close to surfaces (s)	752.87 ± 64.70	753.52 ± 105.20	715.83 ± 81.29	0.07	2, 29	0.932	1.000	0.937	0.950
Frequency of air breathing	23.00 ± 2.62	31.62 ± 7.87	40.50 ± 7.02	2.845	2, 29	0.074	0.525	0.061	0.550

[a]Log-transformed variables; statistically significant values are in bold.

being significantly different. Again, no significant correlation between fight and activity variables could be detected ($P > 0.094$). Levels of F and KT were also uncorrelated ($r = 0.136$, $N = 31$, $P = 0.465$).

Video playback versus one-way mirror

To further test if the behavioral and physiological responses to the live and video stimuli were comparable, a PCA including all behavioral variables and postfight KT and F levels was performed. The first 3 factors of the PCA explained 74.8% of the variance (Supplementary Table 1). Fish from the control group formed a separate cluster but there was a significant overlap between the fish exposed to the live and video playback stimuli (Figure 4). Still, the variability of the response was apparently higher for the video playback group as it showed a larger dispersion of individuals to the group centroid (average distance to centroid: control = 0.574; one-way mirror = 1.0867; video playback = 2.637).

Discussion

Meaningful behavioral and endocrine response to video playback

Results show for the first time in a fish a robust endocrine response to aggression elicited by video images of a conspecific. Plasma levels of KT, the most potent androgen in fish (Borg 1994), increased over 5-fold after observing the opponent on screen and this increase was comparable to the response observed toward the live conspecific fighting behind a one-way mirror. Cortisol also increased significantly after the video playback fight, as previously reported for mirror or paired fights in *B. splendens* (Ramos et al. 2021; Ramos and Gonçalves 2022), while differences to the live stimuli were not significant. This endocrine response was paralleled by the behavioral reaction to the video stimulus. All fish attacked the video conspecific and fights followed a similar sequence toward live and video stimuli, with threat displays being more frequent in the first half of the fight and overt attacks in the second half, as previously described (Vu et al. 2020; Ramos et al. 2021). A PCA combining all the behavioral and endocrine data corroborated the similarity in the response toward the video and live stimuli as there was extensive overlap in the distribution of fish from the 2 groups. Nevertheless, data dispersion seemed to be higher for fish in the video playback group. The reasons for this are not clear but may relate to variation between animals in the sensorial perception of video images (for a discussion, see Fleishman and Endler, 2000).

These results may be compared with previous studies using video playback in *B. splendens*. In the first study, Allen and Nicolleto (1997) showed that males responded aggressively to the manipulated video images of conspecific males of different fin sizes. Displays of the focal animals included the opening of the opercula and other aggression elements, suggesting that indeed video images were being perceived as meaningful. Similar results were obtained by Neri (2019), with males orienting toward a video playback of a conspecific and opening the opercula, the first element to be displayed in a fight (Forsatkar et al. 2016). Interestingly, in the Neri (2019) study, some fish showed only a weak response to the video playback, corroborating our findings that video images produced more variable results than live stimuli. Clotfelter et al. (2006) presented male and female computer-generated animations to assess male and female preference between

Table 2. Descriptive statistics and paired tests of aggressive behaviors of focal fish in response to an opponent fighting a one-way mirror in an adjacent tank, presented live or by video playback

	One-way mirror		Video playback		*t*-Test/ Mann–Whitney *U*		
	N	Mean ± *SE*	*N*	Mean ± *SE*	*t*/ *U*	*df*	*P*
Time with open opercula (s)	8	210.83 ± 37.16	10	222.33 ± 47.69	0.183	16	0.857
Time with fins distended (s)[a]	8	1775.35 ± 12.64	10	1557.93 ± 89.54	49	-	0.424
Time with aggressive color on (s)[a]	8	1775.38 ± 32.48	10	1708.98 ± 124.58	49	-	0.424
Frequency of caudal swings[a]	8	4.625 ± 5.527	10	26 ± 33.269	25	-	0.174
Frequency of charges	8	4.875 ± 9.342	10	22.5 ± 28.96	1.645	16	0.119
Frequency of bites	8	65.875 ± 67.397	10	67.3 ± 110.924	0.032	16	0.975

[a]Differences tested with the Mann–Whitney *U*-test.

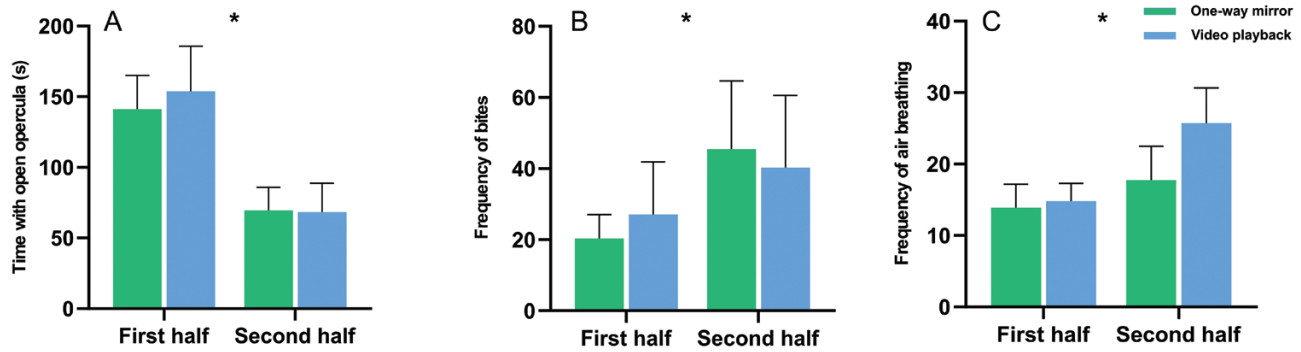

Figure 2. (A) Duration of open opercula, (B) frequency of bites, and (C) frequency of air breathing in the first (0–15 min) and second (15–30 min) halves of the aggression trials. Focal fish were presented with a conspecific fighting a one-way mirror, live (one-way mirror), or as video images (video playback). Mean ± *SE* are shown. * represent significant differences ($P < 0.027$) between the first and second halves of the trial. The live and video stimuli triggered similar responses in the focal fish ($P > 0.407$).

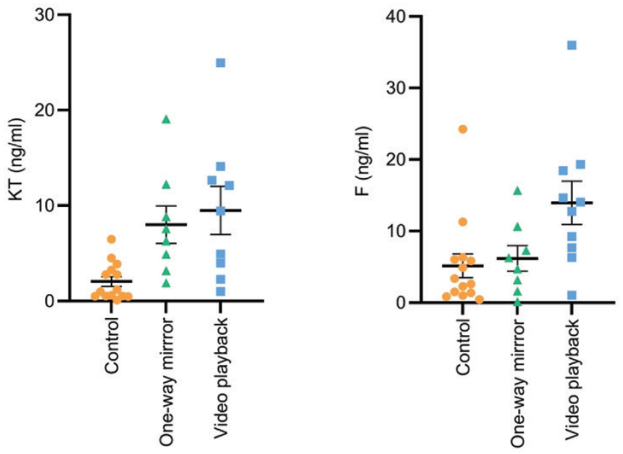

Figure 3. Posttest plasma levels of 11-ketotestosterone (KT) and cortisol (F) in fish presented for 30 min with a conspecific fighting a one-way mirror, live (one-way mirror), or as video images (video playback). Control fish were presented with either an empty tank or with a video playback of the empty tank. Mean ± SE are shown. Different letters represent significant differences ($P < 0.05$) between groups.

pairs of stimuli differing in size or display features. Results suggest that fish were able to discriminate between a pair of stimuli in video playback, but the frequency or duration of aggressive displays was not reported, with only time spent close to stimuli being used as an indicator of preference. In a series of studies, Dzieweczynski and collaborators used video playback to study courtship behavior in male and female *B. splendens* (Dzieweczynski et al. 2014; Dzieweczynski and LaMonica 2016; Dzieweczynski and Kane 2017) further indicating that fish were able to discriminate conspecifics presented in screens. Finally, video playback was combined with mirror tests to investigate differences in aggression between strains of fighting fish (Verbeek et al. 2007), but in this case, no comparison between the response to the video and mirror stimuli was presented. None of these previous studies, however, validated the response of *B. splendens* to video playback by comparing it to an equivalent live stimulus nor provided physiological measures of this response. Taken together, the overall similar response to the live and video playback stimulus demonstrated in the current and previous studies shows that this is a useful technique for the study of aggressive behavior in this species.

The comparison between the 2 experimental groups and the control also demonstrates that males of this species react aggressively and mounts an endocrine response to a noninteractive fight, either live or through video playback. In other words, feedback from the opponent was not needed to trigger the aggressive response. This is relevant because it has been questioned whether measuring the response toward a noninteractive stimulus is appropriate (Bakker and Künzler 1998). Real-life fights are, by nature, interactive and the absence of dynamic feedback from the opponent, and the impossibility to display the full suite of aggressive behaviors (e.g., circling or chases during fights), may generate results that are difficult to interpret. Although this study was not designed to specifically

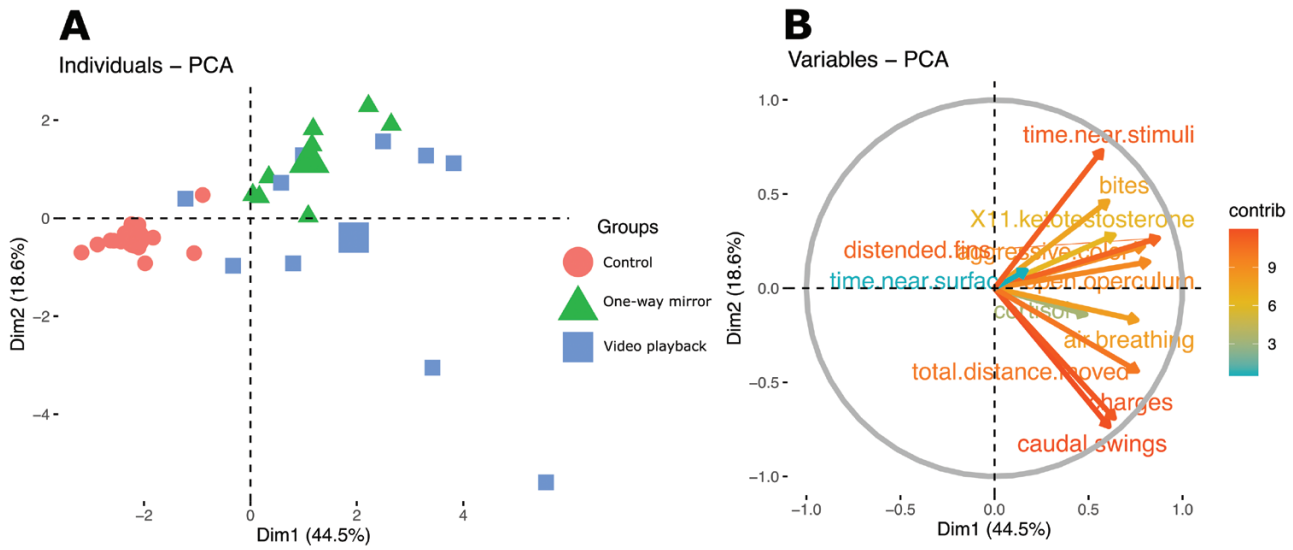

Figure 4. Representation of the first 2 components of a principal component analysis with all the measured endocrine and behavioral variables: (A) individual data—large symbols represent the centroid of the groups; (B) variable loadings—the direction of arrows represents the correlation of variables and size their relative contribution. Numbers in the axis's legend represent the percentage of variation explained by each component.

test the importance of fight interaction for aggressive displays, results can be compared with previously published data from our lab where the aggressive response of males of the same strain, and under similar experimental conditions, were tested with matched-for-size opponents behind a transparent partition (Ramos et al. 2021). While controls in both studies presented a similar frequency of air breathing, interactive fights caused a 2.5- to 3-fold higher frequency of surface air intake as compared with one-way mirror fights, suggesting that they are metabolically more demanding (compare Table 1 in this study with Table 2 in Ramos et al., 2021). The duration of threat displays (average time with opercula open, 211 vs. 372 s) and of attacks (average frequency of bites, 66 vs. 95) was also higher when dynamic feedback was available (compare Table 2 in this study with Table 3 in Ramos et al., 2021). Accordingly, postfight plasma levels of KT (8.0 vs. 10.6 ng/mL) and F (6.2 vs. 13.0 ng/mL) were higher when both males could see each other (compare Figure 3 in this study with Figure 5 in Ramos et al., 2021). Although this comparison supports the hypothesis that aggressive behavior and associated physiological responses are more salient in interactive fights, results should be interpreted with caution as these were separate experiments and uncontrolled differences between studies may explain the results.

Nevertheless, even if the behavioral and endocrine output is reduced toward noninteractive stimuli, video playback and similar assays may still be more appropriate to compare aggression levels across groups as they allow a full standardization of variables, not possible with interactive stimuli.

The role of androgens in aggression

Fish used in our study were isolated for 7 months prior to being tested, thus experiencing a highly stable environment, without social interactions, and a previous work had shown that androgen levels decrease in socially isolated males of *B. splendens* (Ramos and Gonçalves 2022). When exposed to the one-way mirror or video playback challenge after this isolation period, KT levels markedly increased regardless of stimulus type. These results agree with previous data from our lab where plasma KT and testosterone (T) levels increased in male *B. splendens* in response to mirror and interactive opponents (Ramos et al. 2021). This suggests that for *B. splendens* the androgen response to a challenge after a period of social isolation is robust and independent of the type of stimuli. This raises the question of what might be the functional role of the postfight androgen response. A conceptual framework to explain the role of androgens in the regulation of aggression from bird data was proposed in 1990 by John Wingfield and collaborators on what became known as the challenge hypothesis (Wingfield et al. 1990). Under this hypothesis, androgen levels are modulated by the conditions of the social environment, with animals living in more unstable contexts increasing circulating androgens above reproductive levels to facilitate the expression of mating and territorial behavior. This assumption has now been tested in multiple species, usually by assessing the androgen response to a challenge with a live conspecific and comparing it with the stability of the social environment, and a meta-analysis has generally confirmed it in fish (Hirschenhauser et al. 2004; Hirschenhauser and Oliveira 2006). Interestingly, the example cited in this meta-analysis for *B. splendens* was the Dzieweczynski et al. (2006) study where there was no increase in response to the aggression of androgens measured in water. Contrarily, our results show a robust increase in plasma androgens in response to the video playback and one-way mirror opponent in fish that experienced a previously stable environment, supporting this assumption of the challenge hypothesis also for *B. splendens*.

A second assumption of the challenge hypothesis is that androgens facilitate the expression of aggressive behavior during the current fight and/or in future fights. In the Mozambique Tilapia *Oreochromis mossambicus*, plasma KT increases as soon as 2 min after a fight, suggesting a role for androgens as facilitators of aggression during the current challenge. If this is the case, a covariation in postfight androgen levels and aggression might be expected. However, there was no correlation between postfight KT levels and aggressive behavior in response to the video playback or to

the conspecific behind the one-way mirror. This lack of correlation agrees with other studies in *B. splendens* (Ramos et al. 2021; Ramos and Gonçalves 2022) and other fish species (e.g., Desjardins et al., 2006), suggesting that a correlation between postfight circulating androgen levels and the expression of aggressive behavior is weak on inexistent. Alternatively, the androgen increase may prepare fish not for the present but for future fights. Androgens have been proposed to mediate the winner effect, whereby winning a fight increases the probability of winning a future challenge (Oyegbile and Marler 2005). This seems to be corroborated by a study in *O. mossambicus* where blocking androgen receptors canceled the winner effect (Oliveira et al. 2009) and by a study in killifish *Kryptolebias marmoratus* where prefight KT levels correlated with aggression and winning probability (Earley et al. 2013). Interestingly, winning is not always needed to trigger the winner effect as fish fighting their mirror image, where there is no winner experience, may also increase the probability of winning future fights (Dijkstra et al. 2012). Thus, it seems possible that the androgen increase after a fight experience may prepare fish for future challenges, increasing the probability of winning those challenges. However, while T administration has been shown to induce a moderate increase in the duration of opercula opening in *B. splendens* (Forsatkar et al. 2013), castration, which reduces circulating androgen levels, failed to inhibit aggressive behavior in this species (Weiss and Coughlin 1979).

In conclusion, our results support the assumption of the challenge hypothesis that androgen levels increase in more unstable social environments but not the assumption that the function of this increase is to facilitate the expression of aggressive behavior. Further studies manipulating prefight androgen levels and testing the aggressive response to standardized stimuli, such as those presented in video playback, are needed to test this second assumption of the challenge hypothesis in *B. splendens*.

The role of corticosteroids in aggression

As compared to controls, plasma F levels increased in focal fish fighting the video playback aggression stimulus but not when fighting the conspecific behind the one-way mirror. An increase in F levels had been previously shown for the same strain and species after mirror and live conspecific fights were staged (Ramos et al. 2021). These results agree with some other studies in fish (e.g., Félix et al., 2020) but not all (e.g., Chang et al., 2012). There was also no correlation between postfight F levels and aggression, as previously found for this species (e.g., Ramos et al. 2021). Taken together, data offers weak or no evidence for F being a facilitator of aggression during the current fight. On the contrary, there is some empirical support for chronic F levels being negatively correlated with aggression in fish, including *B. splendens*. Wild-type *B. splendens*, which are less aggressive than domesticated fighters (Verbeek et al. 2007; Ramos and Gonçalves 2019), have elevated baseline F levels, both in social groups and in social isolation (Ramos and Gonçalves 2022). In lines of rainbow trout, *Oncorhynchus mykiss*, selected for low and high cortisol responsiveness to a stressor, low responders were more frequently associated with a dominant status (Pottinger and Carrick 2001) and more aggressive (Øverli et al. 2004) than high responders. In the same species, short-term F exposure did not influence aggression while long-term treatment inhibited it (Øverli et al. 2002). One possibility is that short-term F increases in response to aggression but decreases in fish that will become dominant. In fact, in subordinate and dominant rainbow trout, F increases rapidly in both participants in a fight, but it starts decreasing in the dominant fish while it continues to increase in the subordinate (Øvrli et al. 1999). The hypothesis that variation in aggression may be related to differences in chronic levels of F may be tested by long-term manipulation of the HPI axis followed by quantification of aggressive behavior in *B. splendens*.

The increase in plasma F levels observed after a fight may relate with its role as a metabolic hormone. One of the functions of F is to modulate glucose-regulation and glycogen-repletion processes, the 2 important pathways for recovery from physical exercise (Mommsen et al. 1999). In *B. splendens*, fighting is metabolically demanding (Alton et al. 2013), as shown by the acute increase in the frequency of air breathing during contests. Cortisol has been shown to respond to exercise in fish (Milligan 1996), further supporting a possible link between F peripheral secretion and energy allocation. Nevertheless, there was no correlation between postfight F levels and activity variables, including the frequency of air breathing, similarly to what was found in previous work with this species (Ramos et al. 2021). A possible explanation may relate to a time lag between F secretion and blood collection time. In the Mozambique tilapia, F increased very rapidly (within 2 min) after a territorial intrusion but this increase was no longer present after 30 min (Félix et al. 2020). If a similar pattern occurs in *B. splendens*, F may peak early in the fight to promote energy availability but not have a direct relationship with the frequency and duration of aggressive displays performed during a more extended period. Clearly, more studies are needed on the temporal variation of circulating corticosteroid levels in the context of aggression in fish and on their physiological function.

The fact that KT increased after the fight in both experimental groups while F only increased in the video playback group may suggest that the corticosteroid response to aggression was more variable than the androgen response. Interestingly, the response to a mirror challenge of fish from the 2 parental strains (1 fighter and 1 wild type) that originated the animals used in this study was consistent for KT but not for F, with F increasing in wild type but not in fighter males (Ramos and Gonçalves 2022). Variability in the F response after exposure to a stressor between a wild-type and a fighter strain, different than those used at our lab, had also been previously reported (Verbeek et al. 2008). It thus seems possible that the difference in the F response to the video and live stimuli could result from spurious variation between fish from the different experimental groups. In particular, evidence suggests that in fish, interindividual variation in basal levels is higher for corticosteroids than for androgens (Félix et al. 2020), which can introduce variation in the response to the aggression challenge. These results further highlight the need to diminish any factor that can introduce additional variability in the assays, whereby video playback can be a useful tool. Taken together, the results confirm that fighting triggers the peripheral secretion of both androgens and corticosteroids in *B. splendens* although the functional significance of this increase remains to be elucidated.

In conclusion, the study demonstrates that video playback is an appropriate tool to study under laboratory settings the intrinsic motivation for aggression in *B. splendens*. The species is a promising model for decoding the role of steroid

hormones in aggressive behavior, and recent developments, particularly the sequencing of its genome (Fan et al. 2018; Kwon et al. 2022), will allow probing in more detail the endocrine pathways modulating variation in aggression in this interesting fish.
Supplementary material can be found at https://academic.oup.com/cz.

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Experiments followed the ASAB/ABS "Guidelines for the treatment of animals in behavioural research and teaching" (ASAB, 2012). The study complied with the ethical guidelines enforced at the University of Saint Joseph and experimental research with this species was approved by the Division of Animal Control and Inspection of the Civic and Municipal Affairs Bureau of Macao, license AL017/DICV/SIS/2016.

Conflict of Interest statement

The authors declare no conflict of interest.

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Author contributions

D.G., D.A., and S.D.C. designed the experiment. S.D.C. ran the behavioral trials and elaborated the video stimuli. A.R. collected blood and run the ELISA assays. D.A. analyzed the data and prepared the figures and tables. All authors contributed to the writing of the manuscript.
Supplementary Material

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