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ORIGINAL PAPER

High variability in aggression and habituation to the mirror assay in ornamental Siamese fighting fish *Betta splendens*

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Abstract

Understanding consistent inter-individual variability in animal behaviour, known as personality traits, is essential for exploring the mechanisms and evolutionary consequences of behavioural diversity. Aggressive behaviour influences survival, resource acquisition, and reproduction, so clarifying individual differences can enhance our understanding of ecological dynamics and improve experimental design accuracy in behavioural studies. In this study, ornamental male *Betta splendens*, a model organism for aggression research, were analysed for intra- and inter-individual variability in aggressive responses to their mirror image—a standard method for assessing aggression in fish—once per week, and their consistency was evaluated over three consecutive weeks There were significant differences in aggressive behaviour across individuals, with coefficients of variation ranging from 29 to 60%. While most fish exhibited the full suite of aggressive displays, some showed no aggressive behaviour, while others only displayed threat behaviours but did not advance to the attacks. The consistency of individual threat and attack behaviours varied, but repeatability was high overall (intra-class correlation coefficients$\geq$0.5), indicating that individual fish have different levels of aggression. There was habituation to the mirror assay, with aggression decreasing significantly by the second week, though the degree of habituation, a form of learning, varied among individuals in some behaviours. Air-breathing frequency correlated positively with aggression behaviours and can be considered an indicator to infer aggression level in this species. These results indicate that inter-individual variation in aggressive behaviour and habituation to repeated testing using the mirror assay should be considered in aggression studies using *B. splendens* and potentially in other species.

Keywords Aggressive behaviour · Intra-variability · Inter-variability · Repeatability · Waning-effect

Introduction

Animal personality can be defined as a consistent inter-individual variation in behaviour and is relevant to understanding ecological and evolutionary behavioural processes, although the evolutionary stability of personality traits has been questioned (Bell 2007; Kaiser and Müller 2021; Koski 2011; Stamps and Groothuis 2010; Wolf and Weissing 2012). The maintenance of different personality profiles in the population has been explained in light of mechanisms such as balancing selection, fitness trade-offs, and social structure, where each personality type confers specific advantages in varying ecological or social contexts (van Oers and Mueller 2010; Wolf et al. 2007). Balancing selection helps maintain genetic polymorphism by promoting the coexistence of different traits within a population, with each trait providing distinct advantages in varying environmental contexts

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(Penke and Jokela 2016). A key example of this is the fitness trade-off between bold and shy individuals, where bold individuals may achieve greater reproductive success but are more susceptible to predation, leading to a shorter lifespan. Consequently, both bold and shy individuals remain in the population (Dugatkin and Alfieri 2003; Godin and Dugatkin 1996; Stamps 2007; Wolf et al. 2007). Balancing selection can be further shaped by social structure, where different behavioural types can be favoured depending on the context. For example, in European bitterling (*Rhodeus sericeus*), aggressive males defend territories and mate with females, whereas non-aggressive males adopt a sneaking strategy to fertilize eggs while the aggressive males are distracted. In less competitive environments, non-aggressive males thrive, wheares aggressive males excel in more competitive contexts. This social dynamic, with both strategies present, supports the maintenance of both traits through balancing selection (Reichard et al. 2004; Smith and Reichard 2005). The realisation of the importance of inter-individual differences in behaviour changed the approach to studying animal behaviour from considering it experimental "noise" in the data to a factor that should be explored (Hertel et al. 2020; Réale et al. 2010; van der Goot et al. 2021). A way to analyse the consistency of behaviours is to evaluate their repeatability, i.e., the proportion of the variance contributed by inter-individual differences (Bell et al. 2009). It has been suggested that repeatability is affected by biotic and abiotic factors, including environmental change (i.e., an individual's capacity to adjust behaviour in response to environmental variability), taxonomic group, sex, and age (Bell 2007; Bell et al. 2009).

Aggressive behaviour is a conserved trait essential for survival in some species and expressed during critical events such as feeding and mating (Hardy and Briffa 2013). The variation in aggressive behaviour may depend on prior experience, genetics, hormonal or environmental factors (Baran and Streelman 2020; da Silva et al. 2021; Haller 2014; Hock and Huber 2009; Martins and Bhat 2014; van Oers et al. 2005). Studies exploring aggression in fish mainly correlate this trait with other behaviours rather than investigating its consistency (Conrad et al. 2011). Inter-individual variability in aggression can be due to different factors; for example, temperature changes and exposure to predation can generate a positive boldness-aggressiveness behavioural correlation (Bell and Sih 2007; Biro et al. 2010; Colchen et al. 2017). It can also vary between different populations of the same species (Álvarez and Bell 2007; Roy and Bhat 2018). Overall, a meta-analysis revealed that aggressive behaviour tends to be repeatable (Bell et al. 2009). Nevertheless, the impact of social experience, as in the "winner-loser" effect, where those who win or lose fights tend to keep winning or losing in repeated challenges, questions how much inter-individual variation in aggressiveness is due to proximate mechanisms or related to previous experience (Briffa et al. 2015; Hock and Huber 2009). In Mozambique tilapia (*Oreochromis mossambicus*), chemical cues released through urine play a decisive role in determining the outcome of aggressive encounters and if urination is stopped by genital papillae constriction, winning becomes random (Keller-Costa et al. 2012).

The Siamese fighting fish (*Betta splendens*) is a model for studying aggression (Simpson 1968), a role that has been strengthened by the sequencing of its genome (Fan et al. 2018; Srikulnath et al. 2021) and recent publications reinforcing its use in research (Lichak et al. 2022; Yue et al. 2022). Different experimental procedures have been used to measure aggression in this species: live fights (Baenninger 1968; Vu et al. 2021), mirror assays (Baenninger 1966; Ramos et al. 2021), video-playbacks (Alex et al. 2022; Allen and Nicoletto 1997) and robotic replicas (Romano et al. 2017). Additionally, some studies have compared aggression levels between fish of wild origin and artificially selected strains, such as fighter lines (Ramos and Gonçalves 2019; Verbeek et al. 2007, 2008), while others have investigated the behaviour of invasive fish, descended from ornamental strains introduced into the wild, and compared them to individuals reared in captivity (Brand et al. 2021). Additional comparisons include aggression between males and females (Ramos and Gonçalves 2019) and across different reproductive stages (Forsatkar et al. 2017). The mirror assay is the most widely used method for measuring aggression because it is more affordable, easy to implement, and ensures ethical standards by preventing injuries and deaths during confrontations. Moreover, it guarantees a fair fight, as each animal confronts an opponent of identical size in its mirror image. It also has the advantage of avoiding post-fight alterations related to injuries or of developing a "winner-loser" effect, which may affect behaviour repeatability (Briffa and Sneddon 2007; Briffa et al. 2015). The mirror assay has been applied to multiple research objectives, including drug testing (Kania and Gralak 2012; Kania and Wrońska-Fortuna 2016; Khoei et al. 2019; Kohlert et al. 2012; Lynn et al. 2007; Oliveira et al. 2022), chemical communication (Baenninger 1968; Colyer and Jenkins 1976; Dore et al. 1978; Ingersoll et al. 1976), territorial responses (Abate 2005), and artificial selection (Ramos and Gonçalves 2019; Verbeek et al. 2007). However, it has been suggested that the mirror assay provides only a partial predictor of a real fight since the fish may show more aggressive displays towards a conspecific than a mirror image (Meliska et al. 1980; Thompson and Sturm 1965). The use of the mirror assay remains a topic of debate, with its effectiveness appearing to vary with species and context (Ashouri et al. 2024; Balzarini et al. 2014; Cattelan et al. 2017; Desjardins and Fernald 2010; Kohda et al. 2019). However, in the Siamese fighting fish, the mirror assay evokes less variable behavioural responses and similar post-fight endocrine responses than interactions with a live conspecific (with a division separating participants) (Ramos et al. 2021; Ramos and Gonçalves 2019). Given the increasing scientific importance of *B. splendens* as a model for the study of aggression and the prevalent use of ornamental lines with the mirror assay, it is relevant to understand the degree of intra- and inter-individual variability and consistency of aggressive displays in this species. The aim of this study was to explore: (1) how aggression varies between individuals (inter-individual variability), (2) how aggression remains consistent within individuals over time (intra-individual variability), and (3) whether aggression is consistently expressed across weeks (repeatability). This was done by measuring aggressive displays of adult males using the mirror assay, once per week over three consecutive weeks. Ultimately, the study aimed to advance the general understanding of aggressive behaviour in fish while enhancing experimental design to measure aggressive behaviour in *B. splendens* and, potentially, in other species.

Materials and methods

Fish

Thirty-two ornamental blue veil tail *B. splendens* adult males of unknown origin but from the same rearing facility were acquired from a local pet shop. The animals were kept in the Centre of Marine Sciences of Algarve (CCMAR) facilities, and all the procedures were carried out by certified staff. The fish were housed in a recirculating system (Aquaneering, Inc., San Diego, USA) with mechanical and biological filters, UV sterilisation, and salt supplementation to ensure water quality. The pH was 7.0 ± 0.5, temperature 27 ± 0.5 °C, conductivity 800 ± 50 µs/cm and photoperiod 14 h light and 10 h dark. Each fish was allocated to a 1.6 L tank, with external white plastic separators between tanks to avoid visual contact with conspecifics. Fish were fed once in the morning with commercial dry food (Zebrafeed, SPAROS, Olhão).

Biometric measurements were made approximately a week post arrival [1.5 ± 0.3 g in body mass (mean ± SD) and 6.9 ± 0.7 cm in total length] (see Supplementary Information, Table S1), with the fish anaesthetised in water containing 200 mg/L tricaine methanesulfonate (MS222), buffered with sodium bicarbonate in a 1:2 ratio (MS222 to bicarbonate) to maintain a neutral pH. The fish had an acclimation period of approximately one month before the experiments were carried out inside the same fish housing room. No injuries were seen during the experiments with the mirror. After the study, the animals were maintained in the facilities where they continued to live and receive care.

Mirror assay

Transparent acrylic aquaria (18 × 10 × 10 cm) with a mirror on one side covered with a removable opaque white partition were used. The aquaria were filled with 1 L of the same dechlorinated freshwater as in the fish housing system. A video camera (Handycam® DCR-SR210, Sony) laterally facing the aquarium recorded the behaviours. Fish were placed in the aquarium for 15 min with the opaque white partition covering the mirror. After removing the partition, the fish were allowed to interact with their mirror image for 30 min. After each observation, the fish were returned to their home tanks, and the experimental aquarium was cleaned and refilled with new water for the following individual. Each fish was observed once per week over three consecutive weeks in a randomised order. All trials occurred after their daily routine feeding. The duration of three weeks was based on standard practices in the field; more extended periods are rarely used since a waning effect has been reported (Baenninger 1966). Additionally, 30-min sessions were used to ensure sufficient time for the entire repertoire of behaviours to be expressed and highlight variations in behaviours across different subjects (Alex et al. 2022). Video recordings of the mirror assays were analysed with Observer® XT v.15 (Noldus, Netherlands).

The aggressive behaviours were categorised into two groups: threatening behaviours that encompassed those in which the appearance of body size increased (“unpaired fins distention”, “opening of the opercula”, and “caudal swing”), and attack behaviour “bite” consisting of direct physical aggression. Unpaired fins distention and opening of the opercula were measured as state events, and their duration was quantified, while the bite was categorised as a point event and quantified as frequency (see ethogram in Fig. 1). The time each fish took to interact aggressively with the mirror from the start of the observation was registered as “latency to aggression”. The species studied, like other Anabantoidei, can take up oxygen from water through skin and gills and atmospheric air through the labyrinth organ (Graham 1997). During aggressive interactions, they perform different behaviours requiring increased aerial oxygen uptake (Castro et al. 2006). Thus, “air-breathing” frequency was also quantified (Fig. 1). The same researcher carried out the behavioural analyses blind to the fish ID and trial. All measured data are provided in the Supplementary Information, Table S2.

It should be noted that the sample consisted of captive ornamental *B. splendens* housed under standardized laboratory conditions. However, factors such as their rearing

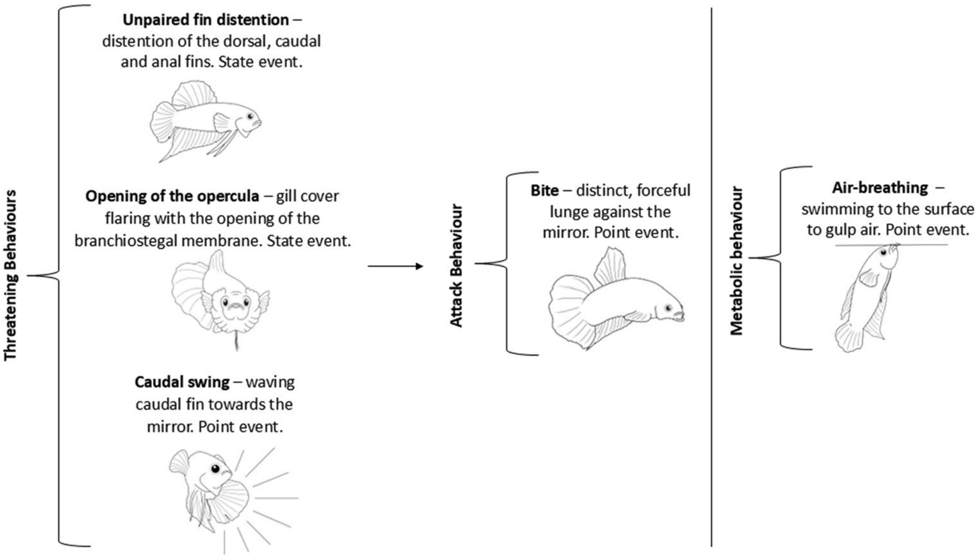

Fig. 1 Schematic representation of the behavioural categories quantified in the mirror assay in *Betta splendens*. Behaviours are grouped into threatening behaviours (unpaired fin distention, opercular opening, and caudal swing), attack behaviour (bite), and metabolic behaviour (air-breathing). The arrow indicates the typical escalation of aggression in *B. splendens*, from threat displays to overt attacks. The vertical line separates metabolic behaviour from aggressive behaviours, as it does not represent a direct component of aggression but is included as a physiological indicator metabolic demand that correlates with aggression

history and prior acclimation to captivity could be a source of variability in their behaviour (Kilkenny et al. 2010; Webster and Rutz 2020).

Statistics

General aggressive behaviour

The means (x̄), standard deviation of the mean (SD), and coefficients of variation (CV) = SD/ x̄ *100) were calculated for each behaviour and individual. Fish ID (1–32) was assigned post-experimentally in ascending order according to the mean bite frequency of each individual (the only direct attack behaviour measured). Spearman correlations with Bonferroni correction (adjusted $\alpha < 0.00179$) were calculated between the mean of the behaviours quantified over the three-week experiment (trials) and fish biometric measurements.

Behavioural clusters

To identify the variables that contributed most to the segregation of the individuals and investigate the existence of different aggression profiles, a principal component analysis (PCA) and a clustering analysis were carried out using the R package Factoextra v1.0.7 (Kassambara and Mundt 2020). The PCA was performed using the mean of each behavioural measure for each individual with scaled data. Clustering was done using hierarchical clustering based on Euclidean distance. A dendrogram was used to group individuals according to their behavioural profiles. The number of clusters (k = 4) was chosen using the Elbow method applied to k-means clustering. A Mann-Whitney two-tailed test with Bonferroni correction was conducted to compare all behavioural variables in the low and high aggression clusters.

Repeatability

A repeatability analysis to determine the intra-class correlation coefficients (ICC = variance among groups/variance within groups + variance among groups) (Boake 1989) was conducted using the iccCounts (v1.1.2) R package (Carrasco 2022). Since none of the variables exhibited normal distribution (Kolmogorov-Smirnov and Shapiro-Wilk tests $P < 0.001$), and because it is not advisable to transform the data to calculate ICC (Nakagawa and Schielzeth 2010), iccCounts was also used to select the distribution that better fitted the variables. State event variables were rounded to the nearest integer as the R package was designed for data counts. This rounding did not substantially alter the original distributions of the variables. The behavioural data were modelled using generalized linear models (GLMs) within the iccCounts R package, which estimates repeatability based on ICC for non-normally distributed count data. The models included 'fish ID' as a random effect to account for individual differences, and 'trial' (experimental week) as a fixed effect to assess temporal variation. The best-fitting distributions were selected separately for each behavioural variable based on: (1) goodness-of-fit, (2) non-significant dispersion and zero-inflation tests (Monte Carlo simulations, $P > 0.05$), and (3) lowest Akaike Information Criterion (AIC) values. The Negative Binomial 1 (nbinom1) distribution provided the best fit for the frequency of "bite" and "caudal swing," as well as the duration of "unpaired fins distention" and "opening of the opercula." The Negative Binomial 2 (nbinom2) was the best-fitting model for the frequency of "air-breathing" (see Supplementary Information,

Table S3). Although both are based on the negative binomial distribution, nbinom1 assumes a linear relationship between the variance and the mean, while nbinom2 assumes a quadratic relationship, allowing better adaptation to different patterns of data dispersion.

Habituation

Friedman's one-way repeated measures ANOVA was used to evaluate habituation to the mirror assay over time. Differences were compared using the Wilcoxon signed-rank post hoc test for multiple comparisons (two-tailed). Data were expressed as the mean and 95% confidence limits. To explore potential inter-individual variation in habituation across trials, two GLMMs were fitted using the glmmTMB (v1.1.5) package (Brooks et al. 2017). The first model included only a random intercept (Variable ~ Trial + (1| ID)), accounting for differences in baseline behaviour across individuals. The second model included both a random intercept and a random slope for trial number (Variable ~ Trial + (1 + Trial| ID)), allowing individual trajectories of behavioural change over time. These models were compared using a likelihood ratio test ($\alpha = 0.05$) to determine whether individuals differed significantly in how their behaviour changed over time.

Statistical software

IBM SPSS Statistics v.26 was used for statistical tests (e.g. Mann-Whitney, Friedman's ANOVA, Wilcoxon signed-rank and Spearman correlation) and RStudio (v4.2.2) (R Core Team 2022) was used for cluster and repeatability analysis and graphical representations with the ggplot2 (v3.4.1) package (Wickham 2016).

Results

General aggressive behaviour

B. splendens usually initiates aggressive displays through threats (unpaired fin distention, opening of the opercula, and caudal swings), transitioning later without reversal to overt attacks (bites). However, not all individuals transitioned to the attack phase during the trial, and some were not responsive to the mirror image (Fig. 2). Inter-individual variability in aggression was high for all behaviours analysed, with unpaired fin distension having the smallest (29%) and caudal swing the largest (60%) CV. Air-breathing frequency was the only behaviour that all fish performed, and it also had high inter-individual variability (CV = 43%) (Fig. 2). Although *B. splendens* is known for its aggressiveness, some animals consistently expressed low levels of aggression (Fig. 2, e.g., fish ID 1 and 2), whereas others were consistently highly aggressive (Fig. 2, e.g., fish ID 32). Latency to aggression was higher in the less aggressive individuals, as shown by the negative correlation with unpaired fin distention, opening of opercula and caudal swing, but not with biting (Table 1).

Air-breathing correlated positively with all measures of threats and attacks ($\rho \geq 0.769$, $N = 32$, $P < 0.00179$) and negatively with latency to aggression ($\rho = -0.662$, $N = 32$, $P < 0.00179$). The highest positive correlations were between the duration of the opening of the opercula and unpaired fin distention ($\rho = 0.879$, $N = 32$, $P < 0.00179$), followed by caudal swing frequency and opening of the opercula duration ($\rho = 0.871$, $N = 32$, $P < 0.00179$). Total length and body mass did not exhibit significant correlations with any of the variables analysed ($N = 32$, $P > 0.00179$) (Table 1).

Behavioural clusters

A cluster analysis was conducted using a Principal Component Analysis (PCA) followed by hierarchical clustering visualized via a dendrogram. The first two components of a PCA explained 86% of the variance, with 70% explained by PC1. PC1 reflects the large inter-individual variability in aggressive behaviour. Less aggressive individuals with higher latency are located on the left side of the plot, while those exhibiting higher aggression levels are on the right side (Fig. 3A). PC2 primarily distinguishes biting frequency from threatening behaviours. Among the behavioural variables, biting frequency contributed most significantly to the principal components, while latency to aggression contributed the least (Fig. 3A, Supplementary Information Table S4 and S5).

The dispersion of the individuals along the axis matches the clusters identified in the dendrogram. The dendrogram identified two main clusters, each integrating two smaller subclusters containing individuals of similar aggression levels (Fig. 3B). The blue cluster in Fig. 3 includes the most aggressive individuals, and the red cluster is the least aggressive. The grey and yellow clusters contain intermediately aggressive individuals, with grey being closer to lower aggression and yellow closer to high aggression. The blue cluster seems to include the fish that attacked more, while the ones that threatened more are in the yellow cluster. All the behavioural variables differed significantly between the two main clusters (unpaired fin distention $U = 11$, the opening of the opercula $U = 2$, caudal swing $U = 16$, bite $U = 42$, air-breathing $U = 3$. latency to aggression $U = 30$, $N_1 = 18$, $N_2 = 14$, $P < 0.001$) (Fig. 3).

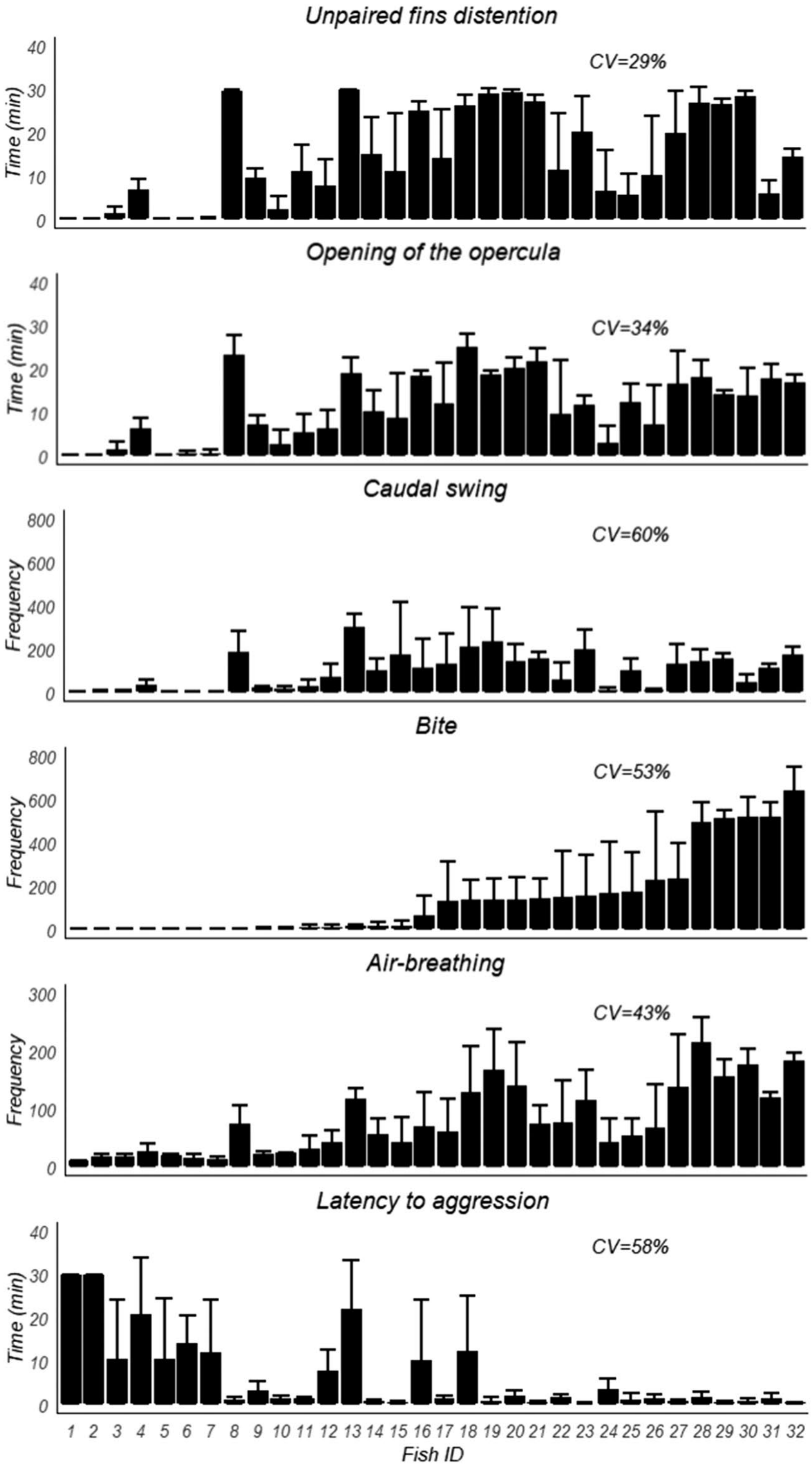

Fig. 2 Individual variability in behaviours towards the mirror image. Data are expressed as mean ± SD of the three weeks measurements in each individual, $N = 32$. The coefficient of variation of each variable for all fish is indicated as CV

Repeatability and habituation

All behavioural variables exhibited high repeatability ($ICC \geq 0.5$). Unpaired fins distention duration had the highest repeatability ($ICC = 0.875 \pm 0.107$) while air-breathing had the lowest ($ICC = 0.466 \pm 0.082$) (Table 2). Only the latency to aggression remained almost unchanged over the three weeks. In contrast, all the other behavioural parameters showed a reduction in the second week compared to the first (further reduced in the 3rd week for caudal swing; $P < 0.05$), suggesting a degree of habituation with repetition (Fig. 4). The comparison of the models (Variable ~ Trial +

Table 1 Spearman bivariate correlations between behaviours and biometric measurements. Data corrected with bonferroni, $N=32$. Values in bold indicate a statistically significant correlation ($P<0.00179$)

	Behavioural data						Biometric measurements	
Spearman Bivariate Correlation Analysis (ρ)	Unpaired fins distention (min)	Opening of the opercula (min)	Caudal swing (frequency)	Bite (frequency)	Latency to aggression (min)	Air breathing (frequency)	Body mass (g)	Total length (cm)
Unpaired fins distention (min)	1.000	**0.879**	**0.832**	0.497	**-0.685**	**0.821**	-0.349	-0.271
Opening of the opercula (min)		1.000	**0.871**	**0.581**	**-0.755**	**0.834**	-0.294	-0.221
Caudal swing (frequency)			1.000	0.501	**-0.722**	**0.769**	-0.281	-0.275
Bite (frequency)				1.000	-0.399	**0.832**	-0.578	-0.040
Latency to aggression (min)					1.000	**-0.662**	0.131	0.125
Air-breathing (frequency)						1.000	-0.349	-0.271
Body mass (g)							1.000	0.003
Total length (cm)								1.000

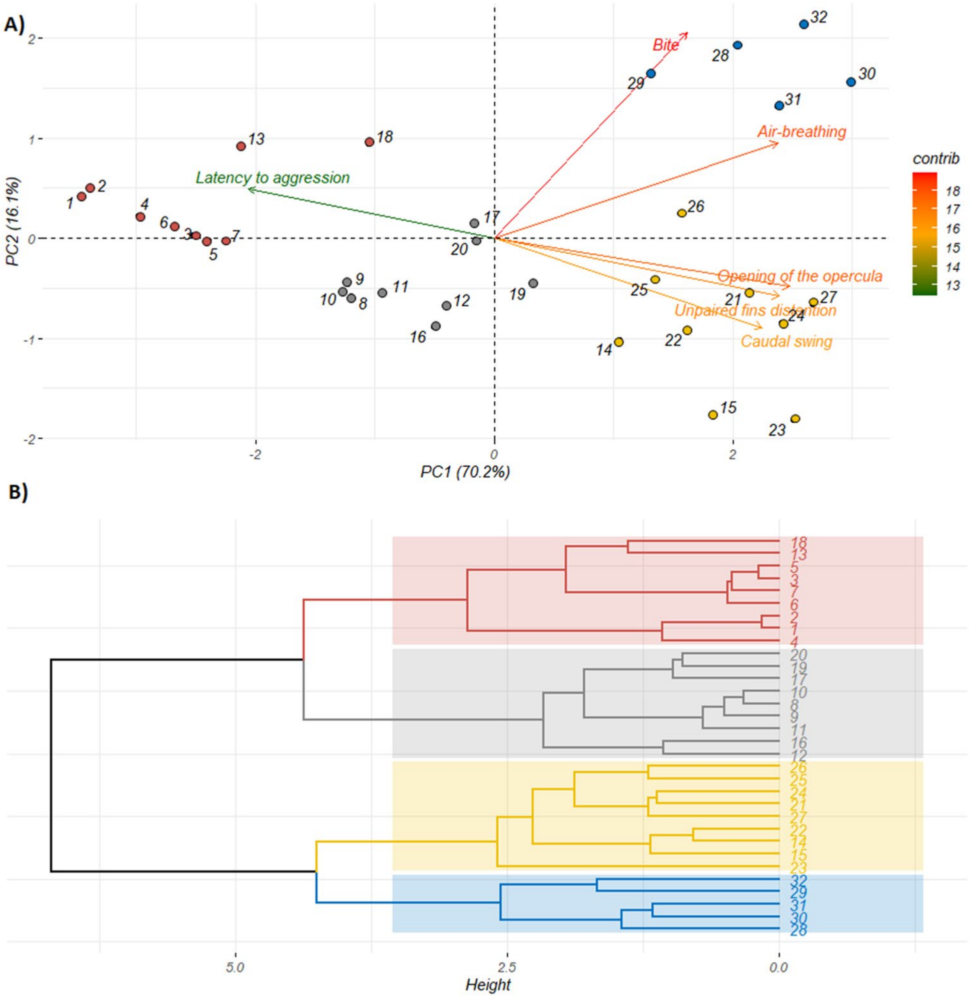

Fig. 3 Cluster analysis of aggressive behaviour in *Betta splendens*. (**A**) PCA 2-D plot with colour gradients arrows indicating the contribution of each variable to the variation. The percentage of variance explained by PC1 and PC2 is shown in parentheses. (**B**) Dendrogram based on Euclidean distances of the mean of the behaviours. Four clusters were identified by the Elbow method and highlighted with the same colours in both panels A and B. Data includes the variables represented in Fig. 1 and "latency to aggression". Each number represents the fish ID, $N=32$

Table 2 Repeatability analysis of the aggressive behaviours using icccounts (v1.1.2) R package (Carrasco 2022) and a general linear model for each variable with “fish ID” as a random factor and “trial” (experimental week) as a fixed factor

Variable	Mean	Variance among subjects	Variance within subjects	ICC	S.E.	ICC 95% CI UL	ICC 95% CI LL	AIC	Distribution model
Unpaired fins distention (min)	17.004	1.413	6.556	0.875	0.107	0.978	0.432	648.225	nbinom1
Opening of the opercula (min)	12.285	1.082	4.604	0.810	0.131	0.954	0.364	614.947	nbinom1
Caudal swing frequency	107.965	1.307	111.360	0.722	0.196	0.937	0.108	940.309	nbinom1
Bite frequency	140.173	1.293	371.621	0.499	0.250	0.833	-0.104	842.289	nbinom1
Air-breathing frequency	81.041	0.582	0.498	0.466	0.082	0.611	0.291	1013.282	nbinom2

Note: ICC, intra-class correlation coefficient; S.E., standard error; ICC 95% CI UL, upper limit of the 95% confidence interval for ICC; ICC 95% CI LL, lower limit of the 95% confidence interval for ICC; AIC, Akaike information criterion; nbinom1, negative binomial 1 and nbinom2, negative binomial 2

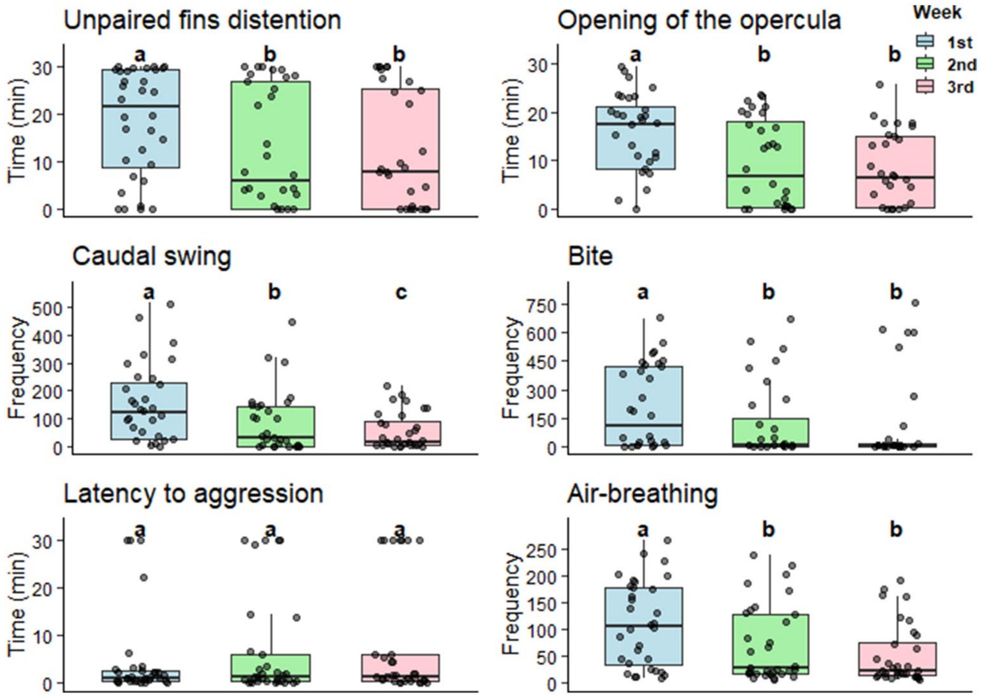

Fig. 4 Variation of individual behaviour over 3 weeks. Friedman’s ANOVA repeated measures with post hoc Wilcoxon signed-rank test comparisons. Different letters indicate a statistical difference between each trial week ($P<0.05$)

(1 | ID)) vs. (Variable ~ Trial + (1 + Trial | ID)) revealed that there is significant variation ($P<0.05$) in individual habituation across trials for the behaviours: unpaired fin distention, opening of the opercula and air-breathing (see Supplementary Information, Table S6).

Discussion

The current study shows that ornamental *B. splendens* have marked inter-individual variability in aggressive behaviour in the mirror assay. This variability was seen in both threats and attack behaviours. The frequency of air-breathing, an indicator of metabolic effort (Castro et al. 2006), was positively correlated with aggressive behaviour. Fish appeared to have distinct aggressive strategies, as each exhibited different proportions of threat and attack behaviours. For instance, some fish spent a significant proportion of the trial engaging in threatening behaviour without exhibiting biting, while others spent only a brief period threatening before transitioning to frequent biting. Data reduction performed with all the behaviours measured supported the existence of inter-individual aggression variability, which could be clustered into low and high-aggressive profiles, with bite frequency as the variable that contributed more to this grouping.

In *B. splendens*, fights start with threatening behaviours, escalating to more aggressive attacks within a few minutes (Forsatkar et al. 2017; Ramos et al. 2021). Although this pattern was observed in the current study, non-responsive fish or fish that did not reach the attack phase were also consistently found. Additionally, no correlation between fish size was found with their aggressive behaviours.

The repeatability results support the idea that there is consistency in the measurements within individuals, as the ICC values were ≥0.5 for all the variables (Delignette-Muller and Dutang 2015). However, interpreting ICC requires careful consideration of variance components. High repeatability arises from high among-individual variation and low within-individual variation. In contrast, minimal differences between individuals or high within-individual variation would result in lower repeatability (Dochtermann and Royauté 2019). High within-subject variability can mask repeatability, which can be the case of caudal swing and bite frequencies. The opposite seems to occur with unpaired fin distention and opening of the opercula durations. Therefore, when variances among and within individuals are similar, it is possible to be more confident about the repeatability as it is less likely to be influenced disproportionately by one source of variability over the other, which in this case happens with the variable air-breathing. Moreover, the standard errors (SE) and confidence interval (CI) provide valuable context by indicating the precision of the ICC estimates. Smaller SE and narrower CI suggest greater confidence in the estimated repeatability, while larger SE and broader CIs imply more uncertainty. In our analysis, we can place the most trust in the ICC values for unpaired fins distention, opening of the opercula, and air-breathing, as these variables show high ICC values with relatively low SE and narrow CI. Conversely, the ICC values for caudal swing and bite frequencies are less reliable due to higher SE and broader CIs, suggesting greater uncertainty in their repeatability estimates.

That aggressive behaviour in *B. splendens* is heterogeneous across individuals is relevant for experimental design. Many studies on this species involve ornamental lines and mirror assays, covering a variety of research areas, but researchers likely use non-paired samples in these studies to avoid the practical complications associated with repeated testing of the same individuals (Baenninger 1966; Brand et al. 2021; Colyer and Jenkins 1976; Ingersoll et al. 1976; Kania and Gralak 2012; Kania and Wrońska-Fortuna 2016; Khoei et al. 2019; Kohlert et al. 2012; Lynn et al. 2007; Verbeek et al. 2007). Despite undergoing significant artificial selection and being maintained in isolation under laboratory conditions where resource competition is absent, these lines still demonstrate consistent repeatability in aggressive behaviour, as demonstrated in our study. To account for the effect of individual differences, a repeated measures design is appropriate with the advantage of reducing the number of animals required (Field 2011). The fact that the fish in our study was of an ornamental strain acquired from a local pet store and their exact ages and origins are unknown could have contributed to the variability. Such factors, including rearing history and acclimation to captivity, may limit the generalizability of our findings to wild populations or other strains of *Betta splendens* (Kilkenny et al. 2010; Webster and Rutz 2020). One possibility is to use fish raised under standardised conditions and with low genetic variability (e.g., inbred lines for the trait under study), and some studies have used this approach (Alex et al. 2022; Ramos et al. 2021; Ramos and Gonçalves 2019).

Habituation to the mirror assay over the three weeks was detected, and variation in habituation was observed for unpaired fin distention, opening of the opercula, and air-breathing. Habituation is a behavioural response decrease due to repeated stimulation and does not imply a sensory adaptation/sensory fatigue or motor fatigue (Rankin et al. 2009). This waning effect has already been explored in this species but with different experimental approaches. Some authors observed consecutive mirror fights with varying recovery periods in between (up to four days) (Baenninger 1966; Clayton and Hinde 1968). The recovery interval in the current study was one week, the longest we found reported in the literature, suggesting that habituation also occurs after longer intervals. Others have described habituation to threat displays over time because of daily exposure to a mirror for up to twelve days (Figler 1972; Meliska and Meliska 1976; Shapiro and Schuckman 1971). Both attack and threat behaviours and air-breathing frequency appear to show habituation. We also found that differences in habituation between individuals exist. Given this, a Latin square experimental design with repeated measures is appropriate for these cases, allowing the distribution of the effect of habituation as each subject undergoes all treatments in all possible orders. This design assigns each treatment to each trial, helping to control for order effects (Keedwell and Dénes 2015). Habituation is a process beneficial for conserving energy in response to repetitive stimuli, focusing on pertinent information while disregarding the irrelevant. Habituation is considered a form of learning, suggesting different levels/rates of learning among individuals (Bell and Peeke 2012; Rankin et al. 2009). To minimise habituation, individuals were housed without visual contact with each other. Notably, latency to aggression remained consistent across experimental weeks, suggesting unchanged motivation despite habituation.

The positive correlation between air-breathing frequency and threats and attack behaviours confirms previous observations (Ramos et al. 2021; Ramos and Gonçalves 2019) and suggests it may be used as an indicator of aggression level. This is linked to the energetic cost of aggressive encounters in this species (Alton et al. 2013). *B. splendens* uses the opening of the opercula as an acute response to defend territory, a behaviour that induces hypoxic stress, and then it is replaced by fin spreading as a chronic response to reduce the energetic costs during the encounters (Forsatkar et al. 2017), in the PCA we identified that both of these behaviours as having similar contributions to define individuals' aggression levels.

The present study contributes to a better understanding of the aggressive behaviour of ornamental *B. splendens* males, showing variation in aggression-related personality profiles. We also show that males habituate to repeated exposure to their mirror image, and variation in how this happens was detected. These results can contribute to optimising experimental design in behavioural studies and suggest that air-breathing frequency might be an easy-to-measure and reliable indicator of aggression. A more comprehensive understanding of aggression development and physiology (including genes, hormones, receptors and associated metabolic cost) is required to dissect individual variability's proximate and ultimate causes.

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Data availability The data supporting this study are provided in the Supplementary Information.

Declarations

Ethical approval All animal experiments, handling and housing were conducted following the ethical standards and guidelines set by the Portuguese "Direção Geral da Alimentação e Veterinária" under the license nº 28660/24-S.

Competing Interests The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this article.

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