

**Lateral bias of agonistic responses to mirror images and morphological asymmetry
in the Siamese fighting fish (*Betta splendens*)**

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Abstract

Behavioural laterality (e.g., during social interactions) is often observed at the individual level in lower vertebrates such as fish, whereas population-level laterality is observed in many higher vertebrates. Population-level laterality can be explained mainly by internal factors (e.g., cerebral lateralization), whereas little is known about the behavioural mechanisms underlying individual-level laterality. Recently, it was revealed that many fish have asymmetrical body morphology, but the relationship between asymmetric morphology and social behaviours has been rarely examined. Here we report the relationship between lateralized eye use during aggressive displays (e.g., body posture) of male Siamese fighting fish, *Betta splendens*, toward their own mirror image and morphological asymmetry. Of 25 males, five exhibited significantly more leftward eye use during left displays, and eight males exhibited predominantly rightward eye use during right displays. Morphological measurement results for the craniovertebral angle and opercular area showed that the craniovertebral angle and opercular area displayed antisymmetry and fluctuating asymmetry, respectively. We found that lateralized eye use during agonistic responses by each fish was associated with the craniovertebral angle, but not with operculum size; lefties (left-curved body) showed mainly left eye use (during left-side displays), and righties (right-curved body)

1 demonstrated the opposite. We suggest that antisymmetric morphologies, such as head
2 incline, are potentially useful for studying the association between cerebral
3 lateralization and individual laterality of behavioural responses. Further, we propose
4 that in fish, morphological asymmetry is related to laterality in various behaviours.

5
6 Keywords: cerebral lateralization, eye use, aggressive display, behavioral laterality,
7 betta, head incline, morphological asymmetry

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13

1. Introduction

Cerebral lateralization is well known among vertebrates, including fish [3, 33, 49, 50].

This lateralization appears to be important for enhancing the performance of complex behaviours. Specifically, it allows the brain to work simultaneously on different tasks

[8, 14, 21, 35] and cope with divided attention [9, 32, 34]. Lateralization has been

assessed in neurological investigations and behavioural experiments. In case of behavioural experiments, preferred limb and eye use in response to a situation can

indicate the degree of cerebral lateralization (reviewed in [49]). Higher vertebrates

mainly show behavioural laterality at the population level (i.e., most or all individuals

of a population share the same bias), whereas lower vertebrates frequently show

behavioural laterality at the individual level (i.e., the direction of bias differs among

individuals, [3]). Behavioural laterality at the population level is considered to be

evolutionarily meaningful; selective pressures for lateral specialization likely operated

within the population [11]. In the case of individual-level laterality, however,

behavioural bias is difficult to examine with respect to cerebral lateralization because

each organism may be subjected to different developmental stressors, such that

information processing differs between each individual, even within a species [2, 3].

Thus far, little is known regarding the purpose and mechanisms underlying behavioural

laterality at the individual level. Additional investigations using several approaches

1 are required to fully understand behavioural laterality.

2 Individual laterality is widespread among fish [3]. Fish eyes are laterally placed
3 on the head, and each eye is exposed to a different visual field. Because visual
4 pathways from each eye project almost exclusively to the contralateral hemisphere [51],
5 transfer of information between the two hemispheres of the brain is low. Therefore,
6 preferences in eye use are considered to be behavioural manifestations of right or left
7 hemisphere specialization in the analysis of incoming visual information [47].

8 In some fish, individual laterality in foraging behaviours is correlated with
9 morphological asymmetry, such as mouth dimorphisms (scale-eating cichlids, [18];
10 shrimp-eating cichlids, [45]; large-mouth bass, [25]). These fish exhibit skewed
11 mouths that open either rightward ('lefty') or leftward ('righty'), owing to asymmetrical
12 joints of the lower jaws to the suspensorium. In addition, lefties have a C-shaped body
13 with more developed left-side muscles and the left side of the fish's head faces forward;
14 righties are exactly opposite [25]. These fish show lateral bias in foraging behaviours
15 that is correlated with morph type; lefties mainly approach from the left side of the prey
16 animal and righties approach from the right side. Correlations between morph type
17 and behavioural laterality of the scale-eater [18] and the shrimp-eater [45] are regarded
18 as adaptations for predation efficiency. Mouth dimorphism is considered to be a

1 genetic trait that is determined by a Mendelian system (one locus, two alleles), with the
2 lefty morph dominant over the righty [18, 20, 38]. Recently, fish of various feeding
3 guilds, e.g., herbivores, benthivores, and piscivores, have been documented to have the
4 same type of mouth dimorphism. It has been suggested that asymmetric morphology
5 in fish is correlated with a variety of behaviours [19]; however, the relationship between
6 asymmetric morphology and behavioural laterality, excluding predation, remains to be
7 clarified, with the exception of disassortative mating [44].

8 Laterality in social behaviours is observed in some vertebrates. Left eye/right
9 hemisphere dominance in eliciting agonistic responses has been demonstrated in many
10 vertebrates, such as baboons [5], chicks [37], lizards [10], and toads [30]. The right
11 hemisphere is suggested to be responsible for the general assessment of novelty.
12 Teleost Belontiidae males show strong agonistic responses at an individual level of
13 lateralization when examining other individuals or their own reflection in a mirror [4, 6].
14 These fish prefer to use either the right or left eye individually in order to assess the
15 opponent. Among Belontiidae fish, the Siamese fighting fish, *Betta splendens*, is
16 highly aggressive in response to mirror reflections, and individuals show marked
17 lateralization [6].

18 Individual laterality in behavioural responses may be explained by the “best side”

hypothesis, which states that fluctuating asymmetry (FA) is related to behavioural bias [13]. Gross et al. [13] demonstrated that male guppies with asymmetric ornamental colours preferentially show their more colourful side to the female, and males with symmetric colours show both sides randomly. In the case of *B. splendens*, operculum size is considered to be important for aggressive and courtship displays, as fish remarkably erect the operculum during these social situations [40]. Therefore, male betta might preferentially display their best side (i.e., larger operculum) to the opponent during aggressive interactions. As a consequence, the fish uses the eye on its best side.

In this study, we examined lateralized eye preference at the individual level during agonistic displays in *B. splendens*. Furthermore, we examined the correlation between laterality in eye preference and morphological asymmetry.

2. Materials and Methods

2.1. Animals

Betta splendens males show intense aggressive behavioural patterns [40], and are widely used as a model species in research on the lateralization of aggressive interactions. For this study, 25 adult male fish were obtained from a local dealer. All experimental males had a similar body size (standard length: 31.33–38.44 mm). Fish

were maintained individually in small plastic containers ($10 \times 10 \times 15$ cm) containing approximately 1 L of aged tap water. Water temperature was maintained at 25–26°C, and artificial light was provided for 12 h per day. The fish were fed dry fish food daily.

2.2.Behavioural tests

An octagonal shaped experimental tank (14.5 cm on a side) lined with mirrors was prepared. The apparatus was filled with water 12 cm deep. One fluorescent lamp (20 W) mounted 1.5 m above the tank provided illumination. The entire apparatus was kept in a darkened room to avoid any influence of the experimenters.

Each fish was introduced into the experimental tank with a small hand net made of fine mesh (10 cm in diameter). First, the hand net that contained the fish was kept in the tank just at the surface of the water for approximately one minute. During this time the fish swam in the net normally. Next, the fish was gently released into the central point of the tank, where it was allowed to swim along the mirrored wall of the octagonal area for 10 minutes. When faced with own its mirror image, males performed aggressive behaviours (displays, dashes, and attempted bites). Above the test apparatus, a video camera (CCD-TR250, Sony) was mounted to record the fishes'

behaviour. Agonistic displays were distinguished from simple shoaling by the presence of specific behaviours such as broadside swimming, dorsal fin erection, and spreading of the opercula. When the fish swam clockwise along the mirrored wall of the tank, it bent its body leftward and displayed the right side of the body (right eye use) and the operculum was towards the mirror; however, in some cases of fast swimming the individual could not assume the adequate pose. The direction of the display (eye use) was then defined as either 'left display' or 'right display', depending on which side of the body was displayed while swimming counter-clockwise or clockwise, respectively. A series of agonistic display was regarded as single aggressive display. The number of left displays (indicating left-eye preference) and right displays (right-eye preference) was recorded using a video camera. When the fish remained perpendicular to the mirror, the data were not used in the analysis. The number of aggressive displays is more appropriate to assess behavioural laterality than is the duration of swimming clockwise or counter-clockwise, because the duration of swimming is likely to include both the time spent in actual aggressive responses and that spent simply swimming. We calculated a laterality index (LI) using the following formula:

$$\text{LI} = \frac{(\text{number of right aggressive displays} - \text{number of left aggressive displays})}{(\text{number of right aggressive displays} + \text{number of left aggressive displays})}$$

We also calculated the mean of the laterality index absolute values to confirm whether the fish showed individual laterality during behavioural responses. Mean absolute indices significantly different from 0 indicated that the fish exhibited individual-level lateral bias [6, 29]. Significant departures from chance (0) were estimated using a one-sample Wilcoxon signed-ranks test.

2.3.Measurement of morphological asymmetries

After the behavioural test, fish were removed from the experimental apparatus and anesthetized using 1/10000 FA-100 (Tanabe Pharmacy, Inc., Tokyo, Japan). The fish were subsequently fixed in a 10% formalin solution for more than two weeks. Bone staining with alizarin red S was performed on the samples for two weeks. The opercula were removed from the body, and the ventral fin was carefully removed under a binocular microscope. These operations were performed in accordance with the Regulation on Animal Experimentation at Kyoto University.

To calculate the asymmetry index (AI) for head incline, we drew two sagittal lines between the parasphenoid and occipital bone and the occipital bone and a three-vertebrae segment of the spine. We then took a digital photograph and measured the angles between the lines using a digital microscope with image analysis software

(VHX-100, Keyence, Osaka, Japan; Fig. 1a). Measurements were taken three times for each individual to reduce observation errors. As measuring the craniovertebral angle of a three-dimensional object is prone to yielding some extreme values, median values were used for the following analysis instead of mean values (in actuality, the measurement errors were small [ANOVA: $F_{2,72} = 148.2$, $p < 0.001$]). Positive angle values were assigned to vertebrae that curved to the right side in the ventral view, and negative values were assigned to vertebrae that curved to the left. Fish with an angle <0 were designated as ‘lefty’, and those with an angle >0 were designated as ‘righty’ (see [25]).

Individual opercular area measurements were taken directly from photos using Image J version 1.42 (US National Institutes of Health, Bethesda, MD, USA; Fig. 1b). The asymmetry index (AI) of opercula was computed for each fish using the following formula:

$$(R - L) / (R + L) \times 100,$$

where R and L are the measured values for right and left opercular areas, respectively.

Frequency distribution characteristics of the two AIs were examined using a Watson test, Shapiro–Wilk test, and Bonett–Seier test for Geary kurtosis.

2.4. Correlation between lateral displays and morphological asymmetry

To assess morphological asymmetries that affect lateral bias during aggressive displays (eye use) in each individual, a generalized linear mixed model (GLMM) analysis was performed. The dependent variable was the direction of the aggressive display (left or right side), and the independent variables were morphological traits (craniovertebral angle and/or AI of the operculum) as a fixed effect and the individual as a random effect.

The GLMM analysis, Watson test, and Bonett–Seier test for Geary kurtosis were performed using R version 2.7.0 [22], and the other statistical analyses were performed using JMP version 5 (SAS Institute Inc., Cary, NC, USA).

3. Results

3.1. Aggressive behaviours

The average number of aggressive displays observed per 10 minutes was 58.2 ± 41.2 (mean $\pm$ SD). The mean LI for aggressive displays did not differ significantly from zero, indicating no overall tendency towards either left or right bias (mean $\pm$ SD = 0.078 ± 0.473 ; one-sample Wilcoxon signed-ranks test: $T = 31.50$, $p = 0.408$). The mean absolute value of LI differed significantly from zero (one-sample Wilcoxon signed-ranks test: $T = 162.50$, $p < 0.001$), which suggests that individual fish are significantly lateralized with respect to agonistic displays (eye use preference). In

other words, individual fish tended to show a bias for either leftward or rightward displays; 13 of 25 fish had a significant bias (five fish showed left bias and eight fish showed right bias; binomial test: $p < 0.05$; Fig. 2). A 1000-times simulation, in which 25 fish randomly displayed 58 times each, showed that the average number of individuals with a significant bias was 1.21 fish (SD = 1.04, binomial test: $p < 0.05$). Therefore, the observed values are higher than those expected from a population in which every fish behaves in an unbiased way relative to a normal distribution.

3.2. Morphological asymmetry of head inclines

The mean craniovertebral angle was approximately 0 (mean $\pm$ SD = $0.638 \pm 2.299^\circ$; one-sample Wilcoxon signed-ranks test: $T = 41.00$, $p = 0.279$). The frequency distribution of the angle strongly deviated from normal (Watson test; $p < 0.01$), and the Geary's kurtosis value was significantly higher than 0.798, which is the value of a normal distribution ($\tau/\sigma = 0.886$, Bonett–Seier test: $p = 0.040$), indicating a clearly bimodal distribution (Fig. 3a). Therefore, the craniovertebral angle was identified as antisymmetry, following the definition of Palmer [26].

The bodies of all sampled fish showed subtle bending to either the left side (C-shaped body) or right side. The craniovertebral angles of nine fish were negative

(lefty), and those of the remaining 16 fish were positive (righty) [25].

3.3. Morphological asymmetry of the opercula

The mean opercular area was 13.10 mm^2 (± 1.31 , SD). As the mean absolute lateral difference ($|\text{right area} - \text{left area}|$) of each individual was 0.32 mm^2 (± 0.21 , SD), opercula on the left and right sides were similar in size (mean $\pm$ SD, $2.48 \pm 1.78\%$ size asymmetry). The mean AI of the opercula was approximately 0 (-0.402 ± 1.483 ; one-sample Wilcoxon signed-ranks test: $T = -52.50$, $p = 0.162$). The frequency distribution of the AI was normal (Shapiro–Wilk test: $w = 0.978$, $p = 0.845$) and its Geary’s kurtosis value did not differ from a normal distribution ($\tau/\sigma = 0.797$, Bonett-Seier test: $p = 0.980$, Fig. 3b). Therefore, opercular area was identified as showing fluctuating asymmetry [26].

A positive correlation between the craniovertebral angle and the AI of the opercula was observed ($R^2 = 0.46$, $F_{1,24} = 19.89$, $p < 0.001$, Fig. 3c). Therefore, fish with greater left curvature tended to have larger left opercula, whereas fish with greater right curvature tended to have larger right opercula. However, the direction of the craniovertebral angle was not completely consistent with the side containing the larger operculum (eight fish were mismatched).

3.4. *Factors affecting behavioural laterality*

We assessed whether bias in behavioural laterality was affected by two morphological asymmetries using a GLMM analysis. Because of the significant correlation between the craniovertebral angle and the AI of the operculum, we used three models in the analysis: model 1, independent variable = the craniovertebral angle; model 2, independent variable = the AI of the operculum; model 3, independent variable = the craniovertebral angle plus the AI of the operculum. Model 3 was performed to evaluate the effect of the either one morphological factor incorporating the effect of another. In model 1, the craniovertebral angle had a significant effect on behavioural laterality (Table 1). In contrast, the AI of the operculum showed no significant effects in model 2. In model 3, only the craniovertebral angle showed a significant effect. The coefficients of the craniovertebral angle in models 1 and 3 were negative, indicating that lefties displayed mainly with the left side and preferred left eye use in response to the mirror, whereas righties displayed mainly the right side and preferred right eye use. Thus, a lateral difference in the craniovertebral angle is a suitable predictor of behavioural laterality of aggressive displays. However, we found no significant correlation between the magnitude of the LI during aggressive displays and that of the

1 craniovertebral angle (lefties, $F_{1,7} = 1.28$, $p = 0.296$; righties, $F_{1,14} = 0.01$, $p = 0.917$).

2 3 **4. Discussion**

4 Each *Betta splendens* male exhibited a preference for either the left or right side during
5 aggressive displays in response to its own mirror image. This individual lateralization
6 in eye use is consistent with results from previous studies [4, 6]. Cantalupo et al. [4]
7 found that in male betta, the direction and degree of laterality in eye use during
8 aggressive displays observed in the first test was consistent with that of second test
9 performed two months later. Thus, behavioural laterality in social displays of this fish
10 is individually determined. These results suggest that the behavioural laterality of each
11 fish is determined by some internal and/or external factor. One explanation is that the
12 direction of the hemisphere controlling agonistic responses differs in each individual [4,
13 6].

14 Over the last two decades, evidence has accumulated of neuroanatomical
15 asymmetry in various vertebrates (reviewed in [36]) and invertebrates (e.g., [28]),
16 including a nematode [42]. Genetic model organisms allow for the comprehensive
17 study of behavioural laterality [7]. For example, recent work in zebrafish has revealed
18 the genetic mechanisms that control the development of neuroanatomical asymmetries

(reviewed in [15]). Even though behavioural laterality during aggressive responses has been identified in numerous organisms, the regions of the brain controlling the behaviour and whether differences in directionality exist between hemispheres remain unclear. Aggression consists of complex cognitive behaviours, including visual scanning, assessment of the opponent, fin erection, dashing, etc. Therefore, the neural mechanisms underlying aggression may be complex.

Some studies have demonstrated a correlation between the direction of behavioural laterality and visceral inversion [1, 12]. The *fsi* strain of zebrafish, which show reversed anatomical morphologies of the viscera and brain (diencephalon), showed a lateralization bias opposite that of wild-type individuals in two behavioural tests, i.e., eye use during a mirror test and a target approach test [1]. Individual laterality in behavioural responses is widely observed among many fish (e.g., [2, 16, 23, 29, 45, 48]), but the actual frequency of visceral inversion is very low in wild-type individuals, e.g., in medaka (0.35%, [17]) and zebrafish (from about 5%, [27]). This evidence suggests that brain asymmetries based on visceral inversion cannot explain most cases of individual laterality. In contrast to previous hypotheses that stated one mechanism is responsible for lateralization of the diencephalon, Barth et al. [1] introduced the concept that different neuronal mechanisms underlying functional

1 lateralization may exist. Our understanding of the linkage between behavioural
2 laterality and brain asymmetry is rudimentary, and research employing several different
3 approaches involving external morphology may provide important information about
4 this relationship.

5 In the present study, more than half of all *B. splendens* exhibited different degrees
6 of left and right bias during aggressive displays. Some researchers have argued that
7 behavioural laterality has biological advantages. For example, McGrew & Marchant
8 [24] reported that wild chimpanzees that showed stronger handedness were more
9 efficient at fishing for termites than were those that showed incomplete handedness. In
10 addition, lateralized fish performed better than non-lateralized fish in escaping predators
11 while capturing prey because they specialize in monitoring predators mainly with one
12 eye while following prey with the other eye [8]. In another study, lateralized female
13 topminnow showed an enhanced ability to attend to different subjects, finding food, and
14 avoiding harassing males [9]. Two other studies have confirmed the benefits of
15 lateralization in fish. Specifically, lateralized fish were found to school with greater
16 cohesion and coordination and to learn to re-orient themselves in a small space more
17 quickly using geometric and non-geometric cues from the environment [3, 41]. In the
18 case of aggressive responses in *B. splendens*, we suggest that fish with pronounced

1 laterality in aggressive displays may conquer an opponent more frequently as compared
2 with fish that show weak laterality. Under these conditions, the strong advantage of
3 lateralization would predict selection against less lateralized individuals. Further
4 investigation is required to evaluate the relationship between the degree of behavioural
5 laterality and win–loss ratios.

6 No correlation was found between lateral differences in the operculum and the
7 direction of aggressive behavioural bias, indicating that the best-side hypothesis is not
8 supported. Because the left-right differences in opercula were small ($2.48 \pm 1.78\%$
9 size asymmetry), betta may not be able to discriminate the morphological asymmetry of
10 opercula. Even some birds may not discriminate such size asymmetry. For instance,
11 starlings are required discriminate asymmetries at least 10% different in size [43].
12 Gross et al. [13] found that male guppies show the brighter sides of their bodies towards
13 live females, but they do not show any lateral display bias towards a non-living model
14 female. These types of environmental conditions may partially explain the present
15 results.

16 Our results clearly indicate that lateralized eye use during aggressive displays is
17 correlated with asymmetry in head incline. Lefty and righty morphs show mainly left-
18 and right-biased eye use during displays, respectively; however, three fish showed

1 mismatched directions (with significant bias) between the head incline angle and
2 aggressive displays (Fig. 2). Further examinations are necessary to determine whether
3 this mismatch is due to the presence of individuals with a major trend reversal or is an
4 experimental artefact. Previous studies have revealed that laterality in foraging
5 behaviours correlates with morphological asymmetry [18, 25, 45]. Takahashi and Hori
6 [44] found that asymmetric mouth morphology is associated with mating tactics in
7 scale-eating cichlids, which exhibit reproductive pairings between lefties and righties
8 (i.e., disassortative mating). These data suggest that left-right biases in various
9 behaviours correspond to morphological asymmetries. In aquatic systems, lateralized
10 behaviour may play a role in individual fitness [46]. Thus far, the reason for the
11 relationship between behavioural laterality of aggressive displays and morphological
12 asymmetry is not clear; however, a variety of lateralized behaviours must depend on
13 some internal factors, such as the brain and/or motor bias. Morphological asymmetries
14 of the mouth and of head incline appear to be an appropriate external indicator of a
15 lateralized internal factor. Such morphological asymmetry is potentially useful for
16 studying the association between cerebral lateralization and individual laterality of
17 behaviour. Therefore, it is necessary to re-examine the widespread behavioural
18 laterality in fish from the perspective of morphological asymmetry.

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1 Table 1.
2 Results of GLMM analysis on the LI of aggressive displays in *Betta splendens*.

3

Model	Variable	Coefficient	SE	<i>z</i>	<i>p</i>
Model 1	Craniovertebral angle	−0.191	0.088	−2.182	0.029 *
Model 2	AI of opercula	−0.050	0.149	−0.333	0.739
Model 3	Craniovertebral angle	−0.321	0.115	−2.783	0.005 *
	AI of opercula	0.300	0.181	1.658	0.097

4

5

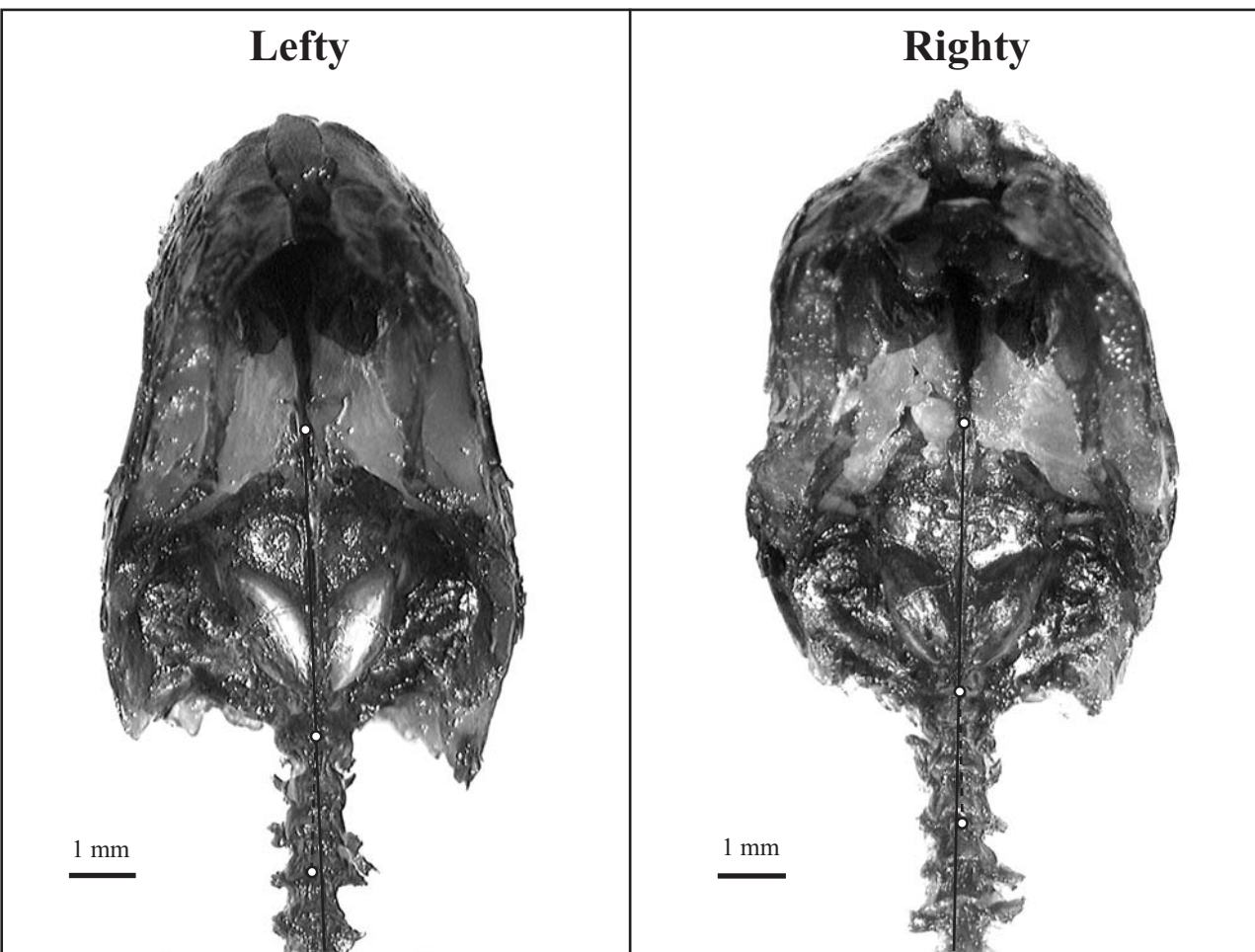
Figure legends

Fig. 1 Photographs illustrating (a) the craniovertebral angle of lefty and righty morphs and (b) left and right opercula in *Betta splendens*. The craniovertebral angle is measured from the intersection of two dorsal sagittal lines: one (solid line) runs from the parasphenoid to the occipital bone, and the other (dashed line) runs from the occipital bone to the three-vertebrae spinal segment in the ventral view.

Fig. 2 Percentage of left-side (empty column) and right-side (grey column) displays for all subjects ($n = 25$). ‘L’ and ‘R’ under the individual number denote lefty and righty fish, respectively. The asterisks above each column indicate significant lateral bias based on a binomial test ($p < 0.05$).

Fig. 3 Frequency distributions of (a) the craniovertebral angle and (b) the AI of the operculum. The dashed line indicates a normal curve fitted to the data. (c) The linear relationship between the craniovertebral angle and the AI of the opercula ($p < 0.001$).

a)



b)



Fig. 1

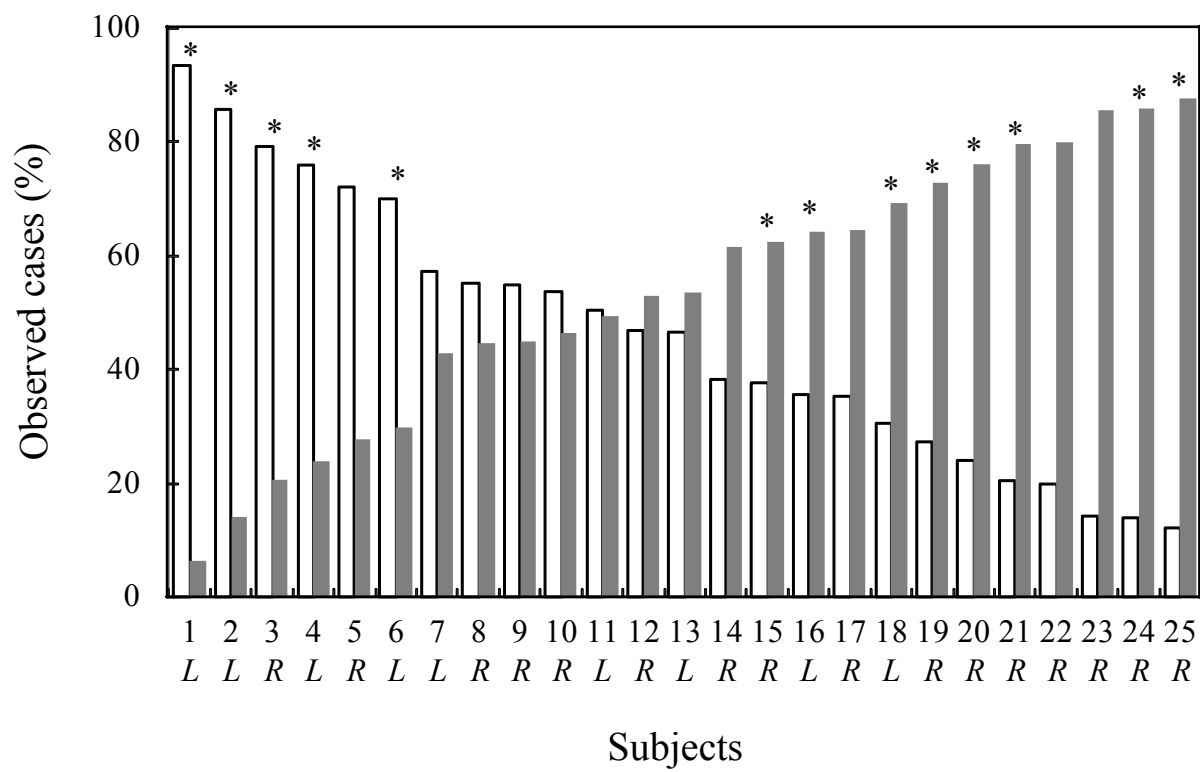


Fig. 2

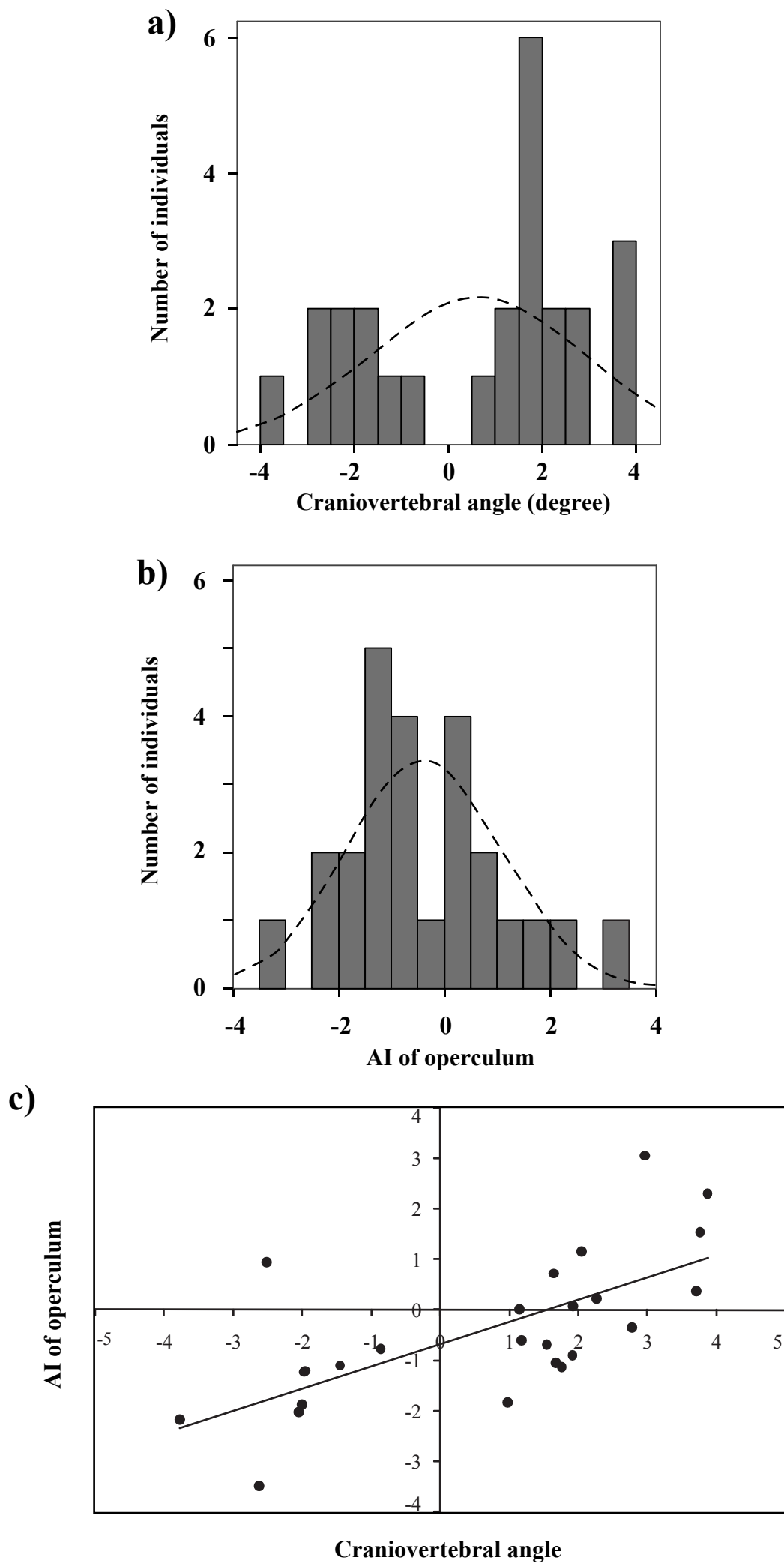


Fig. 3