

BRIEF COMMUNICATION**The angular position of a refuge affects escape responses in staghorn sculpin *Leptocottus armatus***

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The effect of the presence and angular position of a refuge on the direction and kinematics of mechanically-induced escape responses was observed in staghorn sculpins *Leptocottus armatus* using high-speed video. The results showed that the angular position of the refuge did not affect locomotor performance (speed and acceleration), although it did affect the escape trajectories. Therefore, the angular position of a refuge can modulate the direction taken by the *L. armatus* during the early stages of their escape response and this response can be affected by both repulsive (*i.e.* threats) and attractive (*i.e.* refuges) points of reference.

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Most fish species avoid predation by performing an escape response, *i.e.* a fast acceleration burst away from the threat (Domenici & Blake, 1997). Typically, escape responses have been divided into three main stages based on kinematics, corresponding to the first body bend (stage 1), the second body bend (stage 2) and a variable stage involving continuous swimming, coasting or braking (stage 3) (Domenici & Blake, 1997). Fast-start escape responses of fishes are generally mediated by Mauthner cells (control neurons located in the hindbrain of most fish species; Eaton *et al.*, 2001), which, upon stimulation, fire an impulse to the contra-lateral muscle, causing the fish to bend into a C-shape in a direction away from the threat (Domenici & Blake, 1997; Eaton *et al.*, 2001). The kinematics of the first two stages have been studied extensively because they are considered crucial for avoiding ambush predator attacks (Webb, 1976; Walker *et al.*, 2005). By comparison, although stage 3 may be crucial for determining the fish's position after the execution of stages 1 and 2 and hence the likelihood of successful

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escape, this phase of the anti-predator behaviour of fishes has drawn less attention (Domenici & Blake, 1997). While early work had considered escape responses highly stereotypic, more recent work has highlighted that the Mauthner cells participate in a parallel network whose activation produces a C-start motion with a high degree of flexibility rather than stereotypy (Eaton *et al.*, 2001). Hence, not all C-starts look the same, since there can be many modulating factors (Domenici, 2010). In addition, considerable intra and inter-individual variation occurs in escape behaviour and performance (Hitchcock *et al.*, 2015).

Escape responses of animals can be affected by their physical surrounding, such as the presence of obstacles (Eaton & Emberley, 1991) and refuges (Cooper, 1997; Kramer & Bonenfant, 1997; Zani *et al.*, 2009). The advantage of having a refuge nearby lies in the increased protection from predators. Accordingly, reaction distance was found to increase with distance to a refuge in various species of fish (Dill, 1990; Gotanda *et al.*, 2009). Little is currently known, however, about the effect of refuge position on the kinematics and trajectories of the fish escape response. In the absence of a refuge, most animals tend to escape in a direction away from the threat (Domenici *et al.*, 2011). The presence of refuges is known to affect the escape trajectories of some terrestrial vertebrates such as the lizards *Eumeces laticeps* and *Uta stansburiana* (Cooper, 1997; Zani *et al.*, 2009). In other taxa, such as marine invertebrates (blue crab *Callinectes sapidus*), the presence of a refuge can lead to an escape trajectory that is intermediate between escaping towards a refuge and away from a threat (Woodbury, 1986).

Here, a coastal benthic species, the staghorn sculpin *Leptocottus armatus* Girard 1854, was used to test the hypothesis that the escape response in fishes (both kinematics and trajectories within stage 2 and stage 3) is modulated by the presence and the position of a refuge. In the laboratory, *L. armatus* are known to escape away from a threat (Paglianti & Domenici, 2006). In nature, *L. armatus* typically rest motionless on the bottom of the seafloor and hide behind objects or inside shelters (Moyle & Cech, 2000).

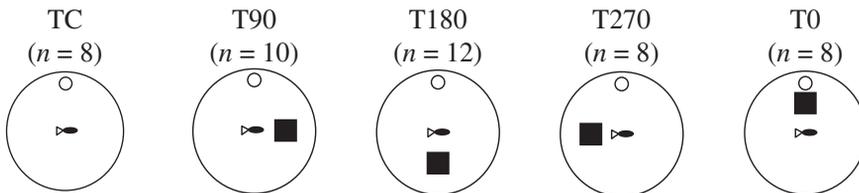
Leptocottus armatus were collected during August 2011 using beach seining off San Juan Island, Washington, U.S.A. Individuals were maintained in four aquaria of 60 × 120 × 30 cm (width × length × depth) that received a constant flow of sea water (salinity 34) at ambient water temperature (12–13° C). *Leptocottus armatus* were held for 3–4 days in ambient light and exposed to a natural day–night light cycle (48° 30' N) without feeding. Sixty-seven *L. armatus* (mean ± s.d., total length, $L_T = 14.2 \pm 0.8$ cm, mass 41.4 ± 6.8 g) were used for the experiments and were tested individually and only once. Twenty-four hours before the experiments were performed, 10 to 15 *L. armatus* were transferred to an acclimation tank that was constructed exactly the same as the experimental tank. The animal care and sampling protocol were approved by the University of Washington in accordance with Institutional Animal Care and Use Committee standards (permit no. 4238-04).

The experimental tank was a round tank 106 cm in diameter and 40 cm high (water depth 20 cm) with white plastic covering the bottom and wall. All of the experimental set-up was surrounded by a black plastic sheeting except for the top, where the camera, lamps and stimulus were located. To test *L. armatus* behaviour in response to predators, a mechanical stimulus was used to simulate an aerial predator attack (Dadda *et al.*, 2010). The stimulus was a 50 ml plastic centrifuge tube (with V-shape bottom, diameter 30 mm, length 115 mm) that was filled with weights (total mass 205 g). The stimulus

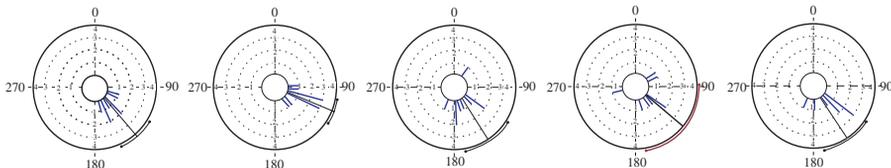
was set to be dropped from a height of 136 cm, by attaching it to a stand. Its release was controlled by switching off an electromagnet. The point where the stimulus hit the water surface was 5 cm from the tank wall.

A 10 cm diameter section of PVC pipe 12 cm long was cut in half lengthwise and used as refuge. The same refuges used in the experiments were provided also in the holding tanks. Preliminary observations showed that *L. armatus* readily occupied the refuges in the holding tanks and spent most of the time inside them. If outside, they would re-enter the refuge when disturbed. During the experiment, the refuge was positioned with its length perpendicular to the wall and the outer end at a distance of *c.* 15 cm from the wall. The position of the refuge was randomized between one of four orientations, 0, 90, 180 or 270° (Fig. 1, treatments T0, T90, T180 and T270). A fifth treatment was performed as a control (TC) where no refuge was provided. As the outer edge of the refuge was 15 cm from the tank wall, the stimulus in treatment T0 hit the water behind the refuge (*i.e.* at 5 cm from the tank wall), from the perspective of the *L. armatus*, requiring the *L. armatus* to escape towards the threat if it was to enter the refuge.

(a) Treatment



(b) S2 escape trajectory



(c) S3 escape trajectory

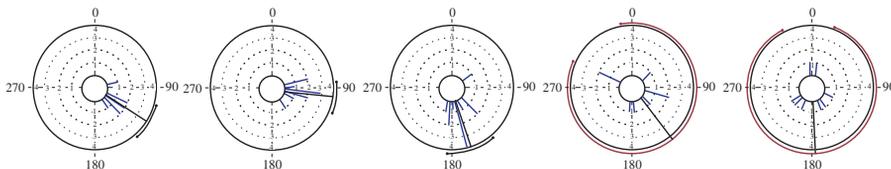


FIG. 1. The escape trajectory distributions relative to the threat (0°) and a refuge in different positions. (a) Four treatments with refuge at 0° (T0), 90° (T90), 180° (T180), 270° (T270) and control treatment (TC). (b) Escape trajectories at the end of stage 2. (c) Escape trajectories at the end of stage 3. Each concentric circle represents a frequency = 1. Each bin is 10° . The mean angle of escape trajectory and its angular deviation is shown for each treatment. Circular graphs are arranged in 5 columns, each one referring to the treatment indicated in the top row, respectively.

[Correction added on 19 April 2017, after first online publication: The bottom right circle has been replaced due to missing average angle line near 180° and the caption has been edited.]

A Fastec Imaging Troubleshooter high-speed video camera (www.fastecimaging.com) operating at $250 \text{ frames s}^{-1}$ with 480×420 pixels, mounted at a height of 210 cm was used to record *L. armatus* behaviour. Two 150 W (2250 lm) halogen lamps positioned above the tank were used to provide illumination. Experiments were conducted during the day from 0800 to 2000 h. The day before the test, individuals were marked with two small pieces (1×0.5 cm each, one on each side of the dorsal fin) of reflective tape (3M ScotchLite; www.3m.com) at 35% of the L_T from the tip of the snout [the centre mass of the *L. armatus* when stretched straight (Paglianti & Domenici, 2006)]. The procedure took <10 s and no anaesthesia was applied. Marking was done by gently pressing on the dorsal side of the *L. armatus* with the tape held by tweezers. The tape attachment lasted only 1–2 days and then the tape fell off without any consequence for the *L. armatus* and their behaviour did not appear to be affected by this procedure. *Leptocottus armatus* were given 24 h to recover before the experiments were carried out.

For the test, individual *L. armatus* were transferred from the acclimation tank to the experimental tank. *Leptocottus armatus* were allowed to acclimate for 25 min in the refuge (in which they remained without being forced) placed in one of four evenly spaced positions (T0, T90, T180 or T270, Fig. 1) near the edge of the wall. At the end of this period, the refuge and the *L. armatus* were gently moved to the centre of the tank where they were left for another 5 min. In order to standardize the orientation of the *L. armatus* to the stimulus, the refuge was positioned perpendicular to the stimulus and the *L. armatus* was always facing the same direction for all the tests (Fig. 1). At the end of this 5 min period, the refuge was lifted from above and repositioned in the original location used at the beginning of the acclimation period. In most cases, *L. armatus* remained still after removal of the refuge. In cases where *L. armatus* moved upon removal of the refuge, the trial was discarded. Fresh sea water was supplied continuously, except after the refuge was moved, preventing any effect of current on *L. armatus* behaviour. The stimulus was then released within 30 s. For the control, a single *L. armatus* was placed in the centre under a refuge oriented perpendicularly to the stimulation and left there for 30 min. After this period, the refuge was lifted and the stimulus released within 30 s.

The following definitions for the fast-start stages were used (Domenici & Blake, 1997). Stage 1 (S1) begins at the first detectable reaction of the *L. armatus* by the anterior part of the body (snout to the centre of mass) and ends when the motion of the anterior part of the body reverses direction. Stage 2 (S2) begins at the end of stage 1 and ends with the next reversal of the turning of the anterior part of the body. Stage 3 (S3) denotes the swimming behaviour after stage 2 until the *L. armatus* stopped swimming or entered the refuge. In the case of the control (no refuge), stage 3 was considered over when the *L. armatus* stopped swimming. The following variables were analysed: responsiveness, *i.e.* the proportion of *L. armatus* that responded to the stimulation, in any given treatment; distance–time performance (speed and acceleration) was based on the analysis of the centre of mass of the *L. armatus* when stretched straight (Webb, 1976; Domenici & Blake, 1997); maximum speed and maximum acceleration were calculated as the highest speed and acceleration, respectively, achieved between the beginning of stage 1 and the end of stage 2; average velocity was calculated as the mean speed achieved during stage 1, stage 2 and stage 3. The duration of stage 3 (Time of S3), as well as the total duration of the escape response (Total time) from the initial reaction until the end of stage 3, were measured. The distance covered (D_C) by the centre of mass

during a fixed time corresponding to the mean \pm s.d. duration of stage 1 plus stage 2 (0.056 ± 0.031 s) (D_C S1 & S2) and during stage 3 (D_C S3) were measured.

Data were smoothed prior to analysis of the maxima, based on the five-point smoothing polynomial fit (Domenici *et al.*, 2008). Escape trajectories were calculated as the angular difference between the initial orientation and the orientation at S1, S2 or S3 stage as in Domenici & Blake (1993). Responses to stimuli from the left and right were pooled as if the stimulus was always on the right side of the animal (Domenici & Blake, 1993). The escape trajectory potentially spanned 360° and therefore was considered a circular variable (Domenici & Blake, 1993). The escape trajectory was determined for each *L. armatus* by measuring the angle between the midline of the *L. armatus* (snout to the centre of mass) and the stimulus direction (*i.e.* the line between the centre of mass at frame 0 and the position where the stimulus hit the water). Hence, an angle of 0° represented a *L. armatus* heading towards the stimulus and an angle of 180° a *L. armatus* swimming directly away from it. Stage 2 (S2) escape trajectory was determined by measuring the orientation of the midline of the *L. armatus* relative to the stimulus direction at the end of stage 2. Stage 3 (S3) escape trajectories were measured by determining the midline orientation relative to the stimulus direction at a point when the snout reached a distance from the wall (D_W), calculated as the mean \pm s.d. distance between the entrance of the refuge and the wall ($D_W = 27.24 \pm 2.18$ cm). *Leptocottus armatus* that did not respond to stimulation were excluded from the kinematic and trajectory analysis. Recordings of *L. armatus* escape behaviour were analysed using an automated tracking programme (WINanalyse, Mikromak, Berlin, Germany; www.mikromak.com). *Leptocottus armatus* were held until the markers fell off and were then released.

For each treatment, a Rayleigh test (Batchelet, 1981) was used to test for randomness of the directions of escape trajectories. Within stages S2 and S3, escape trajectories were compared using the Mardia–Watson–Wheeler (MWW) non-parametric test (Batchelet, 1981). Multiple comparisons were carried out between pairs of treatments (MWW-test) for the S2 or the S3 stage. The MWW-test is a non-parametric test of circular statistics to compare two or several samples and suitable for angular variance comparison. It tests the null hypothesis that the samples came from the same population. The difference could occur in the mean angle, in the angular variance or in both measures.

In this study, a total of 46 individuals out of 67 responded to stimulation, with 8, 10, 12, 8, 8 individuals having responded to the stimulation for TC, T90, T180, T270, T0, respectively. *Leptocottus armatus* rarely moved when initially put into the tank. There were some minor variations, however, in terms of the *L. armatus* orientation relative to the refuge, leading to a real mean \pm s.d. position of $3.58^\circ \pm 9.25^\circ$ (range from -11.99° to 18.17°), $91.60^\circ \pm 23.90^\circ$ (57.09° – 121.95°), $183.10^\circ \pm 13.37^\circ$ (159.41° – 199.17°), $275.00^\circ \pm 14.56^\circ$ (252.37° – 294.22°) for T0, T90, T180, T270, respectively. The clustering was deemed sufficiently narrow for the angles of the treatments, with large gaps between each range, to justify the consideration of refuge location relative to the *L. armatus* as a categorical variable. The percentage of *L. armatus* that responded to each treatment did not differ among the five treatments ($\chi^2 = 3.78$, d.f. = 4, $P > 0.05$). Maximum velocity (ANOVA, $F_{4,41} = 2.041$, $P > 0.05$) and maximum acceleration (ANOVA, $F_{4,41} = 1.604$, $P > 0.05$) for escape responses in the five treatments was not significantly different among treatments (Table I).

Similarly, there were no differences (ANOVA, $P > 0.05$) in total time, time of stage 3, escape distance of stages 1 and 2 (D_C S1 & S2), escape distance of stage 3 (D_C S3)

TABLE I. Maximum velocity, acceleration and proportion of fish entering the refuge for escape responses in the four treatments with refuge at 0° (T0), 90° (T90), 180° (T180), 270° (T270) and control treatment (TC) (mean ± s.d.)

	TC	T90	T180	T270	T0
V_{\max} (m s ⁻¹)	1.19 ± 0.48	0.78 ± 0.34	1.06 ± 0.43	1.21 ± 0.44	1.23 ± 0.40
A_{\max} (m s ⁻²)	23.92 ± 9.09	18.46 ± 5.62	21.58 ± 8.92	25.01 ± 9.73	27.43 ± 8.32
Entering refuge (%)		75	70	12.5	25

[Correction added on 19 April 2017, after first online publication: The acceleration unit has been amended and the term “body flexure” removed from the caption.]

nor average velocity among the five treatments (Table II). For S2, escape trajectories of all treatments were significantly different from random ($P < 0.05$ for T270; $P < 0.001$ in all other treatments). For S3, only trajectories TC, T90 and T180 were significantly different from random ($P < 0.001$ in all cases), while no differences from random were found for T0 ($P > 0.05$) and T270 ($P > 0.05$) (Fig. 1).

Significant differences were found among treatments for both S2 and S3 escape trajectories ($W = 15.83$, $P < 0.05$ and $W = 35.18$; $P < 0.001$, respectively). For S2 escape trajectories, multiple comparisons yielded significant results for the pairs T90–T180 ($W = 10.34$; $P < 0.05$), T90–T270 ($W = 6.77$; $P < 0.05$) and T0–T90 ($W = 7.83$; $P < 0.05$). For S3 escape trajectories, multiple comparisons yielded significant results for the comparisons between TC–T0 ($W = 11.21$; $P < 0.05$), TC–T90 ($W = 8.66$; $P < 0.05$), TC–T180 ($W = 12.47$; $P < 0.05$), TC–T270 ($W = 7.95$; $P < 0.05$), T0–T180 ($W = 7.21$; $P < 0.05$), T0–T90 ($W = 12.78$; $P < 0.05$), T90–T180 ($W = 14.98$; $P < 0.05$), T90–T270 ($W = 7.76$; $P < 0.05$) (Fig. 1). The proportion of fish that ended up in the refuge was significantly affected by the orientation of the refuge ($\chi^2 = 11.1$, d.f. = 3, $P < 0.05$, Table I).

The results show that the presence and the angular position of a refuge did not have any effect on responsiveness and locomotor kinematics, but affected S2 and S3 escape trajectories.

The absence of an effect of refuge on swimming performance may be in part due to the fact that the stimulus had equal strength (same distance between stimulus and *L. armatus*) in all five treatments and therefore posed a similar level of danger in all cases.

TABLE II. Total time, time of stage 3, escape distance covered of stage 1 and 2, escape distance covered of stage 3 and mean velocity in the in the four treatments with refuge at 0° (T0), 90° (T90), 180° (T180), 270° (T270) and control treatment (TC) (mean ± s.d.)

	TC	T90	T180	T270	T0
Total time (s)	0.72 ± 0.32	0.62 ± 0.39	0.87 ± 0.42	0.74 ± 0.45	0.94 ± 0.43
Time of S3 (s)	0.66 ± 0.33	0.56 ± 0.38	0.80 ± 0.43	0.69 ± 0.49	0.89 ± 0.02
D_C of S1 & S2 (m)	0.03 ± 0.01	0.03 ± 0.01	0.02 ± 0.01	0.03 ± 0.01	0.03 ± 0.01
D_C of S3 (m)	0.38 ± 0.19	0.32 ± 0.18	0.31 ± 0.10	0.37 ± 0.12	0.40 ± 0.12
V (m s ⁻¹)	0.65 ± 0.40	0.64 ± 0.25	0.44 ± 0.21	0.70 ± 0.36	0.54 ± 0.31

[Correction added on 19 April 2017, after first online publication: The term “body flexure” has been removed from the caption.]

Swimming performance within the first two fast-start stages, stage 3 and the whole escape response until the refuge was reached was not affected by treatment (Table I). Previous work shows that although speed was not affected by distance-to-cover in the cichlid *Melanochromis chipokae* Johnson 1975, they appeared to choose a combination of flight initiation distance and escape velocity that ensured they reached cover with a constant temporal margin of safety (Dill, 1990). Here, no difference was found in the duration of stage 3 among treatments, most likely because *L. armatus* that escaped away from the stimulus and a refuge positioned near the stimulus (T0 and T270) did not make a long detour to reach the refuge, but settled far away from the refuge. Similarly, the treatments had no effect of swimming performance. Therefore *L. armatus* did not manoeuvre at the expense of swimming performance (Domenici & Blake, 1991) to reach their refuges in any of the treatments.

Escape trajectories were affected by the angular position of the refuge and this effect was already present at the end of stage 2. Specifically, the trajectories for T90 were at a smaller angle (*i.e.* closer to 90° than to 180°) and with smaller variation in direction than those found in T180 and in T0, suggesting that *L. armatus* can modulate their course of swimming in order to direct it towards a safe area within the first two stages of the escape response. Although the effect of refuge on escape direction has already been found in other species, *e.g.* lizards (*E. laticeps*, *U. stansburiana*; Zani *et al.*, 2009) and *C. sapidus* (Woodbury, 1986), this is the first study to the authors' knowledge that shows the effect of refuges on the early stages of the escape response in fishes.

The effect of the refuge was present at the very early stages of the response, suggesting that the Mauthner cells and parallel reticulospinal neurons [*i.e.* the neurons that are likely to control these stages (Eaton *et al.*, 2001)] provide sufficient flexibility to the system so that any sensory input received prior to the stimulation (*e.g.* the sight of a refuge) will be incorporated into the motor output. This is in line with previous work on the flexibility of escape direction, which shows that walls or obstacles can modulate escape trajectories (Eaton & Emberley, 1991; Serena *et al.*, 2009; Mirjany *et al.*, 2011) and that in certain predator species, the direction of fast start after stimulation can be towards the stimulus itself when this is represented by a prey fallen on the water surface (Wöhl & Schuster, 2007). Flexibility here refers to the fact that the escape responses are not stereotypic as they appear to be modulated to the presence of the refuges, rather than to intra-individual variability (Hitchcock *et al.*, 2015), which was unassessed in this work.

Differences among treatments are even larger in S3 trajectories than in S2 trajectories, with most pair-wise comparisons yielding significance. Hence, in the phase of the escape response immediately following stage 2, the angular position of the refuge has a strong influence on escape trajectory. Interestingly, S3 escape trajectories appear to clearly steer the direction of escape towards the refuge only in treatment T90 and T180. In the other treatments with a refuge (T270 and T0), the distribution of S3 escape trajectories were random. Accordingly, only a small percentage of *L. armatus* entered the refuge in these two treatments (12.5% in T270 and 25% in T0). In T270, although the mean direction of S3 escape trajectory appears similar to that of the control, the angular deviation appears to be much wider and the S3 trajectories include some cases in which the *L. armatus* swam towards the refuge. The large variability of response in T270 may be explained by the fact that reaching the refuge in treatment T270 implied a much larger angle of turn (*i.e.* 180°) from the initial orientation of the *L. armatus*

(i.e. 90° from the stimulus) compared with other treatments (i.e. T90 and T180), which may not be as effective as reaching the refuge in treatments T90 and T180.

The large angular deviation found in S3 trajectories of T0 can be explained by the fact that this treatment provided a situation of conflict, in which the stimulus was coming from behind the refuge. In this case, though S2 trajectories do not differ from those of the control, in stage 3 *L. armatus* show a large angular deviation that includes trajectories away and towards the stimulus. This is in line with previous results on the escape response of *C. sapidus* when startled from the direction of the refuge (Woodbury, 1986). Further work could be aimed at testing if this variety of responses in a situation of conflict between the threat and the refuge is repeatable and therefore individual specific.

In conclusion, the current work shows that escape responses can be modulated by the presence of a refuge. The effect of the refuge is present during the first two stages of the response, with important implications for neural control. Furthermore, the behaviour of *L. armatus* during stage 3 shows that some refuge angular positions have a strong effect in attracting *L. armatus*, while in situations of conflict or when a large angle of turn is required, some individuals may prefer to swim away from the threat instead of towards the refuge. The presence of refuges in different positions is likely to affect prey survival, through its effect on escape trajectories.

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