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Behavioral and neural responses of juvenile crayfish to moving shadows

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SUMMARY

One of the most important decisions any animal has to make is how to respond to sensory cues that suggest an imminent attack by a predator. We measured behavioral and neural responses of juvenile crayfish to moving shadows of different velocities while the animals were searching for food. In all experiments, and independent of shadow velocity, each crayfish produced one of two discrete behavioral outputs: it either tail-flipped backwards by rapid flexion of its abdomen or it immediately stopped its forward locomotion. The probability of each behavioral response was dependent on the velocity of the shadows that were presented. While most animals responded with tail-flips to slow-moving shadows and stops were rarely observed, the number of tail-flips decreased as shadow velocity increased. Tail-flips were almost absent for very fast-moving shadows and stopping behavior became the dominating response. By using a non-invasive technique to record neural activity, we were able to identify the underlying neural circuit that controlled the observed tail-flips. All tail-flips were mediated by activation of the medial giant neurons, which are part of a hardwired neural circuit previously described to produce reflexive responses to tactile stimulation.

Key words: behavioral choice, crayfish, escape, giant neuron, predator.

INTRODUCTION

Escape behavior in crayfish has been a popular choice for neuroethological studies. The underlying neural circuits produce powerful behaviors that can be repeatedly elicited and are characterized by large, identifiable neurons that can be easily accessed in dissected preparations (Wiersma, 1947; Furshpan and Potter, 1959; Zucker et al., 1971; Edwards and Herberholz, 2005). In addition, the escape systems provide a rare opportunity to test the effects of neuromodulators on individual neurons and small neural networks (Yeh et al., 1996; Teshiba et al., 2001; Edwards et al., 2002).

Traditionally, escape behavior in crayfish has been investigated by means of tactile stimulation directed to different body parts, which elicits rapid flexions of the abdomen that thrust the animals away from the point of stimulation. Crayfish are equipped with three neural circuits controlling three different types of tail-flip that differ in response latency and move the animal in different directions (Wine and Krasne, 1972; Wine and Krasne, 1982). The fastest tail-flips are generated by pairs of bilateral command neurons, the medial giant interneurons (MGs) and the lateral giant interneurons (LGs). MGs and LGs have non-overlapping receptive fields and produce reflexive, stereotyped escape behaviors in response to strong tactile stimulation (Wine and Krasne, 1972; Wine and Krasne, 1982; Herberholz et al., 2004; Edwards and Herberholz, 2005). Phasic stimulation of the head and thorax activates the MGs, resulting in a behavioral sequence that rapidly thrusts the animal backwards, whereas stimuli applied to the abdomen evoke LG-mediated tail-flips that thrust the animal upward and forward (Wine and Krasne, 1972) (for a review, see Edwards et al., 1999). In contrast to the LGs, the MGs have been implicated in responses to visual stimuli, although only one published report exists to support this notion (Wine and Krasne, 1972). Traditionally, visually evoked tail-flips have been associated with non-giant (Non-G) circuitry (Wine and Krasne, 1972; Wine and Krasne, 1982). The Non-G circuit lacks giant neurons and is less hardwired, allowing for sensory guidance and predetermination of escape angle and direction. The behavioral output is much less prompt and much more variable compared with the stereotypical giant-mediated tail-flips (Wine and Krasne, 1972). Non-G tail-flips cannot be distinguished by behavioral appearance from tail-flips controlled by the giant neurons, thus additional measurements with implanted electrodes or bath electrodes are required for unambiguous identification (Krasne et al., 1997; Herberholz et al., 2001; Herberholz et al., 2004; Finley and Macmillan, 2002).

When testing the effects of feeding on the excitability of the LGs (Krasne and Lee, 1988), it was found that the LG neurons were inhibited when the animals were eating, while sensory and motor systems were unaffected. This supported the notion that the LGs belong to a set of response-dedicated 'trigger' neurons, i.e. neurons that serve as decision makers for behavioral choice. Several decision makers in the nervous system have since been identified, each responsible for and active during a specific behavior. Mutual inhibition among these decision-making neurons creates a behavioral hierarchy (Edwards, 1991); escape in crayfish is inhibited during backward walking and feeding (Bellman and Krasne, 1983; Beall et al., 1990), and in sea slug escape, swimming inhibits feeding while feeding inhibits withdrawal (Kovac and Davis, 1977; Kovac and Davis, 1980; Jing and Gillette, 2000). More recently, the concept of single decision-making neurons has been expanded by the discovery of large neuron populations that participate in the decision-making process leading to behavioral choice (Kristan and Shaw, 1997; Shaw and Kristan, 1997; Calabrese, 2003). In addition, it has been shown that dissimilar or conflicting behaviors are often controlled by shared neural circuitry (Gillette et al., 2000; Esch and Kristan, 2002; Popescu and Frost, 2002). This suggests that populations of decision makers exist and single decision-making

neurons contribute to a set of different behaviors that are also shaped by the environment (Esch et al., 2002; Briggman et al., 2005).

We found that juvenile crayfish that were exposed to moving visual threat stimuli while searching for food always displayed one of two antipredatory behaviors. Each animal made a discrete behavioral choice by either tail-flipping backwards or interrupting forward locomotion. The frequency of tail-flips was highest when slowly moving shadows were presented, while stops dominated in response to fast-moving shadows. Tail-flip responses were mediated by activity in the MG neurons, which are elements of a neural circuit primarily associated with escape behavior elicited by mechanosensory stimulation.

MATERIALS AND METHODS

Crayfish (*Procambarus clarkii* Girard) were obtained from a commercial supplier (Atchafalaya Biological Supply Co., Raceland, LA, USA) and individually isolated in small water-filled plastic containers (height 10 cm, length 15 cm, width 8 cm) for 1 or 2 weeks before being used in experiments. All animals were fed the same amount of food (Ocean Nutrition Formula One Shrimp pellets; Aqua Pets Americas, Salt Lake City, UT, USA) and were last fed 1 week before being tested. Animals were kept under a constant 12 h:12 h light–dark cycle and all experiments were performed at approximately the same time each day. All animals were thoroughly checked for intactness and no animal was used that had molted 2 days prior to the experiment. Animals that molted within a 2 day period after the experiments were not included in the results. Each animal was used only once.

A total of 92 animals of similar size (ranging from 3.4 cm to 3.6 cm, mean ± s.d. 3.5±0.1 cm; measured from rostrum to tail) were successfully tested in our experiments.

The set-up (Fig. 1A,B) consisted of an experimental tank (height 21 cm, length 31 cm, width 17 cm) separated into different compartments and filled with deionized water to a height of 5.0 cm. The tank was constructed so that water could flow from one end of a narrow tunnel (height 4 cm, length 24 cm, width 5.5 cm) to a 'start compartment' (height 21 cm, length 6 cm, width 12 cm) located at the other end (Fig. 1A). Water was directed into the tunnel using a 0.5 cm diameter polyethylene tube connected to a reservoir. Flow was regulated at a rate of 190 ml min^{-1} by means of a flow meter (Cole-Parmer Instrument Company, Vernon Hills, IL, USA). Water left the tank through a 1.0 cm round opening placed in the start compartment 5.0 cm above the bottom of the tank. The start compartment was separated from the tunnel by a removable barrier. A pair of bath electrodes was attached to the tunnel walls, located 8 cm from the tunnel entrance (Fig. 1A). The inside of the tunnel was painted white and three sides of the tank were covered with white paper. Additionally, the side facing the light source and shadow-generating apparatus was covered with black cardboard (Fig. 1B). A video camera (Canon XL2) was positioned above the tank for recording the behavior. The camera was connected to a TV monitor (Sony WEGA) used to observe the animals during trials (Fig. 1B). A food odor solution was produced by crushing 1.0 g of medium-sized shrimp pellets (Ocean Nutrition Formula One, Aqua Pets Americas), and filtering the extract dissolved in 1 l of distilled water. Stock solution was made fresh every few days and 200 ml of it was diluted in 5 l of distilled water for the experimental solution. Food coloring (red food color; McCormick, Hunt Valley, MD, USA) was used to visualize the flow and five measurements were performed without animals in the tank. The measurements showed consistent flows that arrived at the bath electrodes 13±1 s after they were turned on and at the start compartment 17±3 s later.

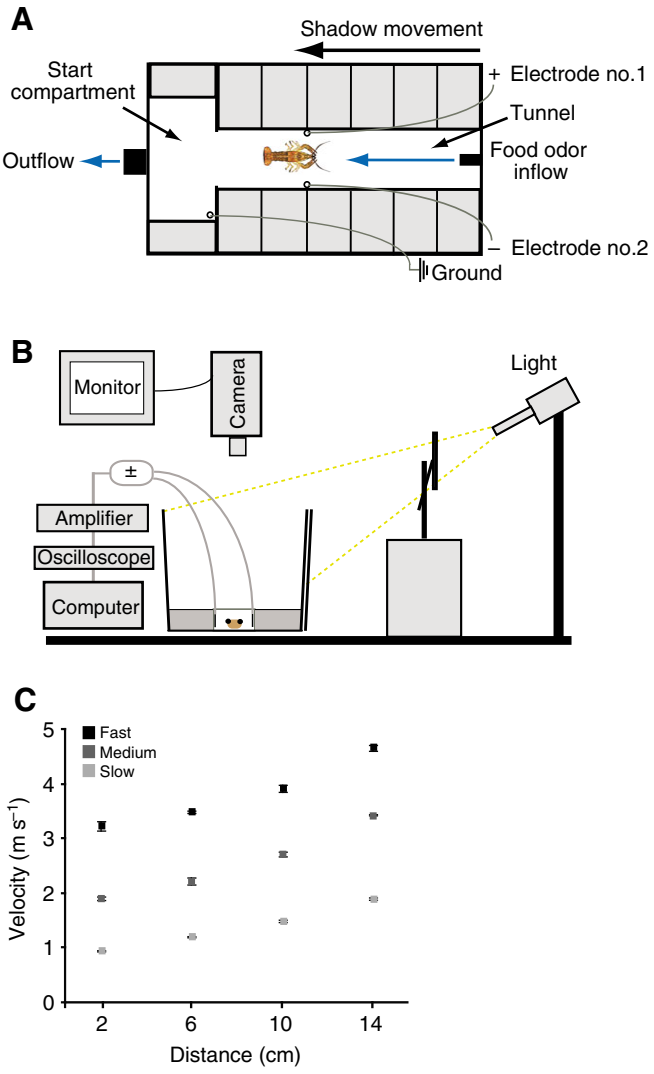

Fig. 1. Experimental set-up and shadow velocities. (A) Top view of the experimental tank. Water containing food odor flows into a tunnel on the right side and exits on the left. Animals enter the tunnel from the start compartment and approach the food odor release point. A pair of bath electrodes is attached to the tunnel walls, 8 cm from the tunnel entrance. The bath is grounded with a ground wire. Shadows always move from right to left over the tank. (B) Side view of the set-up. Animals inside the tank are filmed with a camera positioned above the tank. The camera is connected to a TV monitor. Bath electrodes are connected to an amplifier and an oscilloscope. Signals are recorded on a computer. The shadow is produced by swinging a plastic rectangle through a light beam directed onto the tank. The tank wall facing the light and shadow apparatus is covered. (C) Average shadow velocities and accelerations derived from five repeated measurements with silicone photodiodes placed between the right tunnel wall and the bath electrodes. Velocities and accelerations for all three shadows (slow, medium, fast) are highly consistent as evidenced by small standard deviations.

Shadows were generated by moving a rectangular piece of plastic (15 cm×7.5 cm) through a light beam focused on the experimental tank (Fig. 1B). Crayfish inside the experimental tank were unable to see the goose-neck illuminator (Fiber Lite MI-150; Dolan Jenner Industries, Boxborough, MA, USA) used as the light source or the apparatus that generated the shadow. Brightness inside the tank was measured each day with a lux light meter (SM 700; Milwaukee Instruments Inc., Rocky Mount, NC, USA) before experiments were

started. Brightness was reduced from 200 lx (before shadow) to 8 lx when the plastic rectangle completely covered the light beam. The plastic rectangle was moved by a weighted lever, which was held in place before its release by a trigger mechanism. The trigger was operated manually, and upon release caused the plastic rectangle to swing through the light beam. The speed of the rectangle (and resulting shadows) was changed by altering the weight on the lever and by altering the initial start position of the lever.

Shadows were modeled to move at three changing velocities (Fig. 1C) to most closely resemble shadows that are cast by an attacking predator. Average velocities were based on the fast-start (attack) swim speed of predacious fish (Webb, 1978; Harper and Blake, 1991; Domenici and Blake, 1997). Velocities and accelerations of the shadows were measured with an array of five silicone photodiodes (Allied Electronics, Fort Worth, TX, USA) each spaced 4 cm apart and aligned in the center of the experimental tank. The photodiodes were arranged to cover a distance of 16 cm, ranging from the end of the tank (where the shadows first became visible) to the position of the bath electrodes, i.e. the location where the shadows passed above the animals. The diodes were coupled to an amplifier (A-M Systems, Sequim, WA, USA) and signals were recorded on a personal computer with Axoscope software (Axon Instruments, Union City, CA, USA). Five repetitions were recorded for each shadow and average velocities (between each pair of diodes) were computed from these measurements (Fig. 1C). Shadow movements were extremely consistent for each measurement and were repeatedly confirmed by control measurements during the course of the experiments. Average velocities (between the first and last photodiode) were determined as 1.3 m s^{-1} for slow shadows, 2.4 m s^{-1} for medium shadows and 3.7 m s^{-1} for fast shadows. Faster shadows accelerated more than slower shadows, although the differences were small (Fig. 1C).

The behavioral response to a single shadow exposure was recorded on videotape and electrical recordings derived from the bath electrodes were stored on a computer. Bath electrodes were used as previously described (Herberholz et al., 2001; Herberholz et al., 2004). In short, the two bath electrodes of a pair were placed on opposite sides inside the tunnel to record field potentials generated during tail-flips (Fig. 1A,B). The electrodes were made of copper wire (24 AWG, 0.25 mm insulation except for the tips; Belden CDT Inc., St Louis, MO, USA) and connected to an amplifier (A-M Systems). The bath was grounded using a ground wire. Amplified signals (×1000) were filtered, digitized and recorded with Axoscope software on a personal computer. Identification of giant-mediated tail-flips is warranted by their initial large muscle potentials (mostly due to the simultaneous activation of muscles by the giant motorneurons) and by the immediately preceding giant neuron action potentials. LG- and MG-mediated tail-flips can further be distinguished by clear differences in behavioral appearance. Recordings from Non-G tail-flips lack the giant neuron action potential and the large initial deflection, consisting only of smaller and more erratic muscle potentials (Herberholz et al., 2001; Herberholz et al., 2004; Finley and Macmillan, 2002).

Each experiment was started by transferring a single animal from its home tank into the start compartment and allowing it to acclimate for 10 min. Following this period, the video camera positioned above the tank was turned on, the barrier separating the start compartment from the tunnel was carefully opened and the flow of food odor was started. At this time, the software program that recorded the electronic signals from the bath electrodes was also started. Attracted by the food odor, animals entered the tunnel and walked towards the end where the highest concentration of food odor was present. The movement of the crayfish was observed by watching the camera display on the TV monitor. Crayfish sometimes interrupted their movement while walking towards the source of the food odor. Animals that had stopped near the bath electrodes before shadow presentation were later excluded from the results. In all cases, as soon as the rostrum and eyes of the animal passed the pair of bath electrodes, the plastic rectangle was manually released, thus producing a shadow rapidly moving towards and then passing over the animal. Each animal was exposed to only one shadow, and different groups of animals were exposed to different shadow velocities. All individual compartments of the experimental tank were thoroughly washed between each single experiment.

Unless otherwise stated, data are presented as means and standard deviation (mean ± s.d.). Statistical software (SPSS version 14.0; SPSS Inc., Chicago, IL, USA) was used for analysis and each applied statistical test is specified in the text.

RESULTS

Behavior before shadow exposure

Each crayfish was used in one experiment and was exposed to a single threat stimulus. After the animals were placed into the start compartment, they were allowed to acclimate for 10 min. During this time, crayfish would walk around freely and explore the start compartment. After the flow that contained the food odor was turned on and the barrier that separated the start compartment from the tunnel was opened, one animal failed to enter the tunnel within 600 s. This animal was removed and the experiment was terminated. All other crayfish entered the tunnel and reached the bath electrodes, near the location where they were exposed to the shadows, on average 161±107 s (N=92) after the flow was turned on. Only once did an animal return to the start compartment after entering the tunnel. However, this animal quickly returned to the tunnel and continued its directed search for food. This shows that the concentration of food odor and flow rate used were sufficient to elicit targeted foraging activity. It also shows that all animals were exposed to food odor before they were exposed to the shadows. The behavior of the animals was monitored by means of a TV screen, and shadows were manually triggered as soon as the animals reached the bath electrodes. Shadows were only activated when the animals were in motion.

We applied single-frame video analysis (measured on the TV screen) to determine the position, body orientation and location of each animal inside the tunnel as shown in the last video frame before the behavioral response to the shadows was produced. Positions were assigned as left (L), right (R) and center (C), and body orientations were measured with a protractor in angles that diverged from 0°, a position in which the long axis of the animal's body paralleled the tunnel walls. Deviations to the right and left were assigned negative and positive values, respectively. Most animals moved along the sides of the tunnel and were positioned on the left or right (L 43, R 38) whereas fewer animals were positioned in the middle of the tunnel (C 11). On average animals were orientated at an angle of –0.8±11.1° (N=92), which indicates that their bodies were typically aligned with the tunnel walls. Since manual operation of the shadows was guided by the animals' location tracked on the monitor screen, we used the video recordings to determine the exact location of each animal immediately before the behavioral response to the shadow was generated. All tested crayfish were in very similar locations when exposed to the shadows, on average 0.56±0.15 cm (N=92) past the bath electrodes.

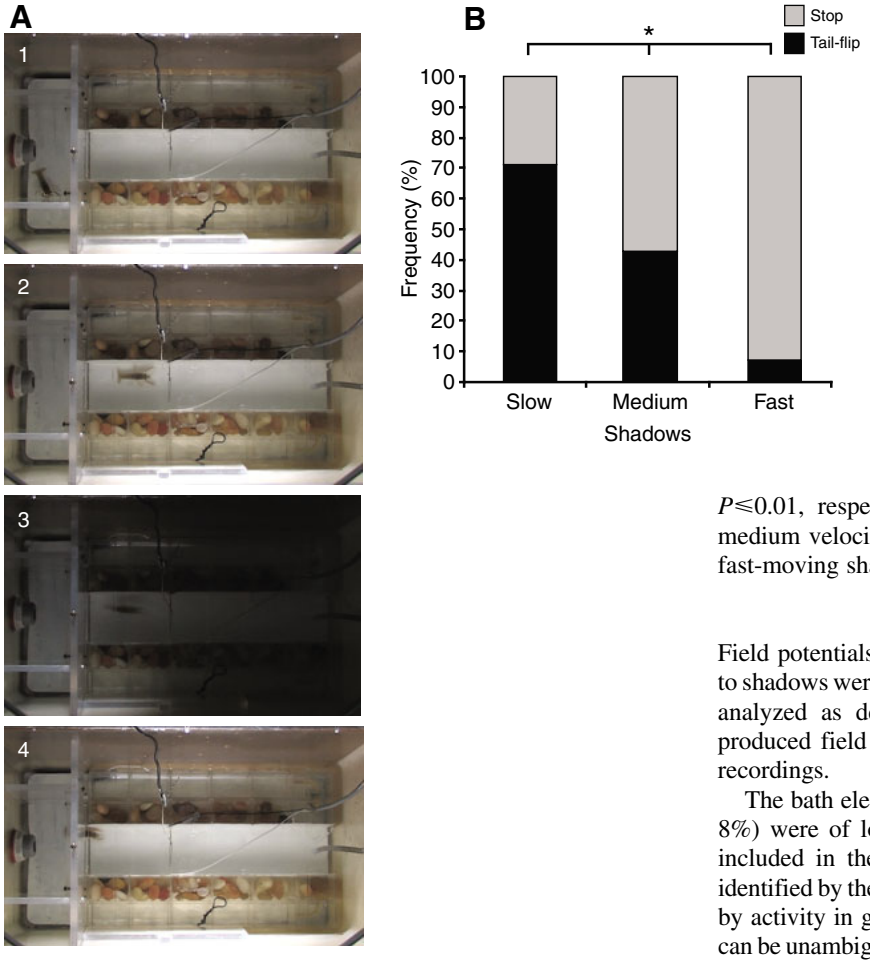

Fig. 2. Example of a tail-flip response and response patterns for different shadows. (A) An example of a tail-flip response of a crayfish exposed to a slow shadow. Shown are (1) the animal in the start compartment shortly after the experiment was started, (2) the animal in the tunnel walking towards the food odor release point and approaching the bath electrodes, (3) the animal producing a tail-flip in response to the shadow and (4) the animal in its final position after completing the tail-flip. (B) Patterns of behavior in response to shadows of different velocities. The number of tail-flips decreases with increasing shadow velocity while the number of stops increases. The differences in response pattern are statistically significant (*$P \leqslant 0.01$).

Behavioral responses to different shadows

Independent of the type of shadow presented, each crayfish (N=92) produced one of two distinct behavioral outputs: it either tail-flipped backwards (N=37) or it immediately stopped locomotion (N=55). None of the animals ever continued forward locomotion without a clear behavioral response (tail-flip or stop) to the shadows.

Fig. 2A shows an example of a crayfish that responded with a tail-flip to a slow-moving shadow. Single frames from the video recording taken during the experiment illustrate the position of the animal in the start compartment at the beginning of the experiment (Fig. 2A, 1), the position in the tunnel approaching the bath electrodes (2), the execution of the tail-flip in response to the shadow (3), and the location of the animal shortly after the tail-flip was produced (4).

When exposed to different shadows (see Materials and methods for description of shadows), each behavioral output was observed at different frequencies (Fig. 2B). In response to slowly moving shadows, most crayfish tail-flipped (71%; N=22) while fewer animals stopped (29%; N=9). Shadows of medium velocity elicited a smaller number of tail-flips (43%; N=13) and more stops (57%; N=17) while tail-flips were almost absent (7%; N=2) and stops were almost exclusively used (93%; N=29) in response to fast-moving shadows (Fig. 2B). The observed patterns of behavioral output expressed in response to the three shadow types differed significantly (Chi-squared test: $P \leqslant 0.01$; Fig. 2B); responses to slow shadows were significantly different from responses elicited with shadows of medium and fast speed (Fisher's exact tests: $P \leqslant 0.05$ and $P \leqslant 0.01$, respectively) and responses observed for shadows of medium velocity differed significantly from responses evoked by fast-moving shadows (Fisher's exact test: $P \leqslant 0.01$).

Neural activity during tail-flips

Field potentials generated by animals that tail-flipped in response to shadows were recorded with the bath electrodes and subsequently analyzed as described in Materials and methods. Stops never produced field potentials of notable size in any of the electronic recordings.

The bath electrode recordings obtained for three tail-flips (N=3; 8%) were of low quality due to high noise levels and were not included in the analysis. All other tail-flips (N=34; 92%) were identified by their distinct electronic signature as tail-flips controlled by activity in giant interneurons (Fig. 3). Giant-mediated tail-flips can be unambiguously identified by the giant neuron action potential that precedes large phasic muscle potentials (Fig. 3A,B). All tail-flips were further classified as MG-mediated tail-flips because they induced a backward motion that is characteristic for MG tail-flips, and also because the only other giant interneurons that control tail-flips (i.e. the LG interneurons) cannot be activated by visual input.

While the initial tail-flip was always mediated by MG circuitry, we occasionally (N=13, 14%) observed tail-flips that followed the first MG tail-flip (Fig. 3B). These tail-flips (up to three; mean ± s.d. 1.4±0.7) moved the animals farther backwards, sometimes as far as into the start compartment. They were produced shortly after the first tail-flip, often within one or two video frames. Since the initial MG tail-flip moved the animal some distance from its original location near the bath electrodes, the tail-flips that followed were more difficult to identify by their electric signature. They were, however, most probably mediated by Non-G circuitry since the recorded field potentials for these secondary tail-flips were more characteristic of Non-G tail-flips (Fig. 3B) and the giant neurons are inhibited during Non-G-mediated 'swimming' that follows the initial tail-flip of an escape response (Kramer and Krasne, 1984; Wine, 1984).

Comparison of stops and tail-flips

Since juvenile crayfish use only two different escape strategies (tail-flip or stop) in response to moving shadows, we decided to analyze possible factors that may influence the behavioral choice (Fig. 4). Although we controlled for hunger state with a rigorous feeding schedule (see Materials and methods), we tested whether animals that tail-flipped could be differently motivated (i.e. being more or less hungry) when moving towards the simulated food source than

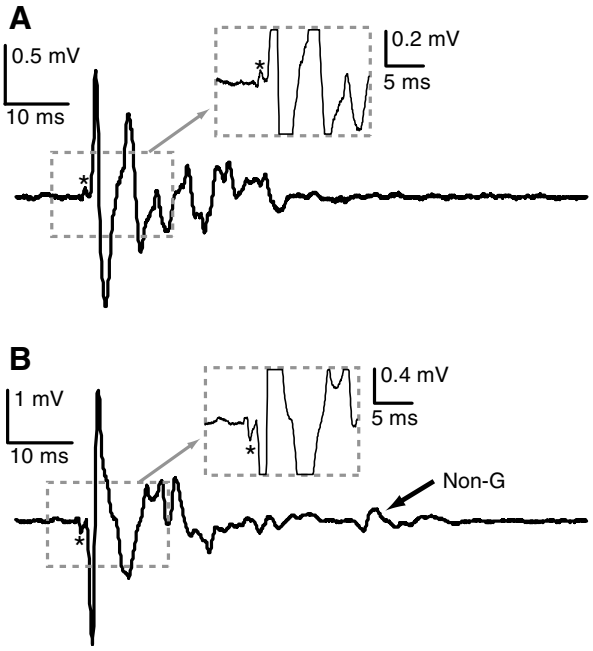

Fig. 3. Electric field potentials recorded with bath electrodes during tail-flips. (A) An example trace of a tail-flip in response to a slow shadow. The action potential of the medial giant neuron (MG, asterisks; magnified in the inset) can be seen and enables non-ambiguous identification of the tail-flip as mediated by giant neuron activity. The large deflections that follow are field potentials generated by simultaneous muscle contractions caused by the giant spike. (B) A second example trace of a tail-flip in response to a slow shadow that is followed 43 ms later by a second tail-flip. The action potential of the MG neuron (asterisks; magnified in the inset) can be seen for the first tail-flip while the much smaller and less phasic potential for the second tail-flip is characteristic of field potentials caused by activity in the non-giant (Non-G; black arrow) circuit. See text for further explanation.

animals that did not tail-flip. To identify possible differences in motivation, we measured the time it took the animals to leave the start compartment and walk to the bath electrodes after the door was opened and the flow of food odor was started. Animals that later stopped in response to shadows and animals that later responded with tail-flips showed very similar times for approach, thus indicating that the behavioral response to shadows was probably not influenced by different motivational states (Fig. 4A). Animals that stopped took on average 163±104 s (N=55) to approach the bath electrodes while animals that tail-flipped spent on average 159±114 s (N=37) before reaching the same position in the tunnel, demonstrating a non-significant difference (Mann–Whitney test: $P \geqslant 0.7$; Fig. 4A).

We also compared the positions, body orientations and locations for animals that made different decisions. The animals' positions inside the tunnel (left side, center, right side) did not differ significantly (Chi-squared test: $P \geqslant 0.3$; Fig. 4B) for animals that employed stops (L 24, C 5, R 26; N=55) or tail-flips (L 19, C 6, R 12; N=37). Body orientations were also very similar and non-significant (Mann–Whitney Test: $P \geqslant 0.6$; Fig. 4C) between crayfish that stopped (–1.2±10.8°; N=55) and crayfish that used tail-flips (–0.1±11.7°; N=37). Finally, the locations (in reference to the bath electrodes) of the animals that stopped (0.55±0.16 cm; N=55) were very similar to the locations for animals that tail-flipped (0.57±0.14 cm; N=37), reflecting a non-significant difference (Mann–Whitney test: $P \geqslant 0.4$; Fig. 4D). Thus none of the measured parameters differed significantly for animals that utilized either tail-flips or stops as a response to the shadows.

DISCUSSION

We found that foraging juvenile crayfish respond to approaching shadows with two discrete and incompatible behaviors; they either immediately stop their forward locomotion or they produce MG-mediated tail-flips that rapidly thrust them backwards. None of the presented shadows were ever ignored.

We observed inter-individual variability among crayfish exposed to the same type of stimulus. For example, in response to shadows that moved at medium velocity, about half the animals chose to tail-flip, whereas the other half produced stops. All tested animals were kept under the same controlled conditions; they were equally sized and were fed an equal amount of food at the same time on the same day. Because they showed very similar latencies for initiating their

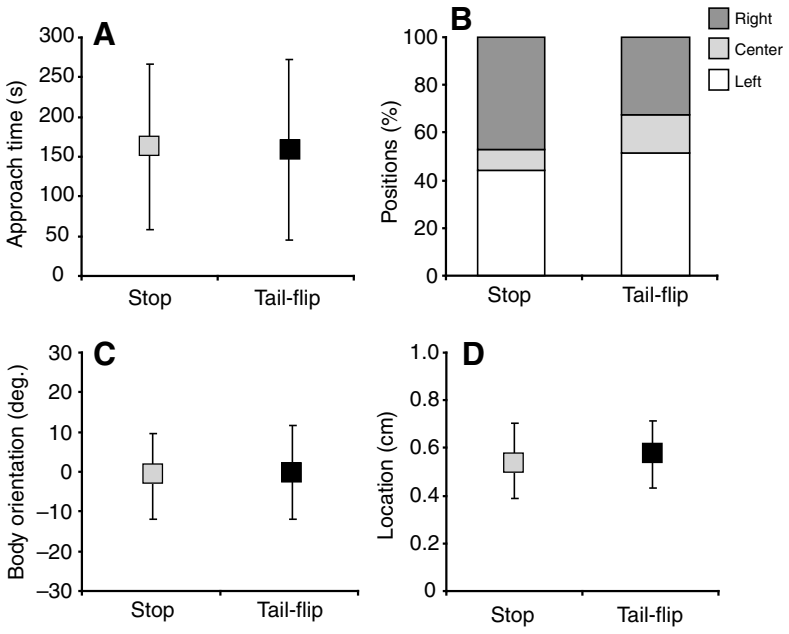

Fig. 4. Comparison between crayfish that stopped and crayfish that tail-flipped in response to shadows. (A) Approach time, i.e. time to reach the bath electrodes after the experiment was started, does not differ between animals that later stopped or tail-flipped when exposed to shadows. Mann–Whitney test: $P \geqslant 0.7$. (B) Patterns of positions inside the tunnel for animals that later stopped or tail-flipped when exposed to shadows are not significantly different. Chi-squared test: $P \geqslant 0.3$. (C) Body orientation does not differ significantly between animals that later stopped or tail-flipped in response to shadows. Mann–Whitney test: $P \geqslant 0.6$. (D) The locations in the tunnel (past the bath electrodes) are not significant different between animals that later stopped and animals that later tail-flipped to the shadows. Mann–Whitney test: $P \geqslant 0.4$.

food search, it appears that animals that tail-flipped and animals that stopped were equally motivated to forage. Since all animals were only tested once using a single shadow presentation, we don't know whether individuals had a predisposition for one defensive behavior or the other and whether this choice preference may also be echoed in other behavioral situations. However, variability in antipredator behaviors is common and has been described in several other model systems including rodents, where the prey's choice of freezing or fleeing in response to an approaching predator is often based on individual differences (Eilam et al., 1999; Edut and Eilam, 2004; Eilam, 2005).

Dichotomous antipredator behaviors have been reported for other invertebrate and vertebrate animals; for example, in response to distant predators, freezing responses often dominate while fleeing is elicited as the predator closes in on the prey (Ranter, 1976; Ranter, 1977; Ydenberg and Dill, 1986). Moreover, threats occurring during inescapable confinement have been shown to cause freezing more than fleeing in several species of mammal (Blanchard et al., 2001).

We found that the number of tail-flips was reduced in favor of stops when shadows moved towards the animals at high velocities. The effects of different predator attack speeds on prey escape behavior have been sparsely studied. One recent report showed that blue tits dodge sideways more often when exposed to a fast-approaching predator model than when attacked at low speed (Lind et al., 2002). In our study, crayfish exposed to fast shadows may have experienced 'inescapable' situations because the high predator attack speeds may have made a timely escape response physiologically impossible. Thus, in response to such inescapable attacks, tail-flipping behavior could have decreased because the associated costs of tail-flipping (e.g. loss of energy, increasing the distance to the food, enhancing visibility) may have outweighed any benefits this escape strategy has over the stopping strategy. This possibility requires further investigation, including measuring the escape latencies of crayfish in relation to shadow positions and testing how crayfish respond to shadows that approach from the back – a situation in which MG-controlled tail-flips could be maladaptive.

Stopping, tail-flipping, and foraging are mutually exclusive behaviors, i.e. they cannot happen at the same time. Backward walking and defense posture inhibit the LG neurons in adult crayfish (Glantz, 1974a; Glantz, 1974b; Beall et al., 1990), and Bowerman and Larimer (Bowerman and Larimer, 1974) described a single descending interneuron in crayfish brain connectives that upon activation suppressed all ongoing movements and froze the animal in position. Therefore, it seems plausible that neurons responsible for temporarily freezing juvenile crayfish inhibit neurons that promote forward walking and the MG neurons; but relevant analyses of the actual circuitry have yet to be conducted.

To our knowledge, neurons that connect visual inputs to the MGs have not been described; a possible consequence of sparse documentation of MG-mediated tail-flips in response to visual stimuli observed in freely behaving crayfish. Prior to this study, MG activity evoked by a purely visual stimulus was only reported once, and shadows were found to be insufficient to reliably evoke MG tail-flips (Wine and Krasne, 1972). Visual stimuli primarily activate Non-G circuitry that produces tail-flips of varying angles and directions (Wine and Krasne, 1972; Wine and Krasne, 1982). Wiersma (Wiersma, 1961) reported giant-mediated responses to visual stimulation but this was before the nature of Non-G tail-flips was recognized; the tail-flips observed by Wiersma were most probably mediated by Non-G circuitry (Wine and Krasne, 1972).

Here we show for the first time that MG escape tail-flips are frequently used as the initial response to moving shadows. Consistent activation of the MGs in response to shadows may be facilitated by a number of factors: the animals were searching for food when stimulated, they were in motion and they were of juvenile stage. Therefore, the motivational state, behavioral state and developmental state of the animals may have affected the thresholds for visually elicited MG tail-flips. The complete absence of Non-G tail-flips as the primary response to moving shadows in our study may be explained by the relatively high stimulus velocities we used; Non-G tail-flips are characterized by longer response latencies than giant-mediated tail-flips, which make them an ineffective escape strategy when rapid responses are required (Wine and Krasne, 1972; Reichert and Wine, 1983; Kramer and Krasne, 1984). The importance of the MG circuit in mediating adaptive escape responses is further supported by two recent reports: the MGs are frequently activated during aggressive encounters between two crayfish when a sudden drop in MG threshold identifies the loser of the fight (Herberholz et al., 2001), and activity in the MG circuit underlies most escape responses when juvenile crayfish are attacked by natural predators such as dragonfly nymphs (Herberholz et al., 2004).

By successfully identifying the MG neurons as major contributors to decision-making in crayfish, we can now take advantage of their accessibility for intracellular physiological experiments. These future investigations will provide a deeper understanding of the neural mechanisms underlying decision-making processes and behavioral choice.

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