

# The effects of head and tail stimulation on the withdrawal startle response of the rope fish (*Erpetoichthys calabaricus*)

Hilary S. Bierman<sup>1</sup>, Julie E. Schriefer<sup>2</sup>, Steven J. Zottoli<sup>4</sup> and Melina E. Hale<sup>1-3,\*</sup>

<sup>1</sup>Committee on Neurobiology, <sup>2</sup>Department of Organismal Biology and Anatomy and <sup>3</sup>Committee on Computational Neuroscience, University of Chicago, Chicago, IL 60637, USA and <sup>4</sup>Department of Biology, Williams College, Williamstown, MA 01267, USA

\*Author for correspondence (e-mail: mhale@uchicago.edu)

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## Summary

While most actinopterygian fishes perform C-start or S-start behaviors as their primary startle responses, many elongate species instead use a withdrawal movement. Studies of withdrawal have focused on the response to head-directed or nonspecific stimuli. During withdrawal, the animal moves its head back from the stimulus, often resulting in several tight bends in the body. In contrast to C-start or S-start behaviors, withdrawal to a head stimulus generally does not involve a subsequent propulsive stage of movement. We examined intraspecific diversity in withdrawal behavior and muscle activity patterns of the rope fish, *Erpetoichthys calabaricus*, in response to stimulation of the head and the tail. In addition, we describe the anatomy of the Mauthner cells and their axon caps, structures that are generally absent in species with a withdrawal startle. We recorded high-speed video (250 Hz) and electromyograms (EMGs) from 12 electrodes in the axial muscle during the behavioral

response. We used Bodian silver staining techniques to visualize Mauthner cell and axon cap morphology. We found that *E. calabaricus* responds with a withdrawal to both head and tail stimulation. Tail stimulation elicits a stronger kinematic and muscle activity response than head stimulation. While withdrawal movement generally constitutes the entire response to head stimuli, withdrawal was followed by propulsive movements when the tail was stimulated, suggesting that withdrawal can both act alone and serve as the first stage of a propulsive startle. Unexpectedly, bilaterality of muscle activity was variable for responses to both head and tail stimuli. In addition, we were surprised to find that *E. calabaricus* has a distinct axon cap associated with its Mauthner cell. These data suggest that the withdrawal response is a more diverse functional system than has previously been believed.

Key words: fast-start, startle, Mauthner, *Erpetoichthys*, withdrawal.

## Introduction

Withdrawal behavior is the primary aquatic startle reaction of many elongate fishes and amphibians. These species span the phylogeny of vertebrates and include lampreys (Currie and Carlsen, 1985, 1987a), eels (Eaton et al., 1977; Meyers et al., 1998; Ward and Azizi, 2001), many teleosts, salamanders and caecilians (Ward and Azizi, 2001). The focus of withdrawal studies has been the response of the animals to head stimuli. During the withdrawal response elicited from the head, the head is moved backward, toward the body and away from the stimulus. Rotation of the head away from the stimulus may occur early in the response (Meyers et al., 1998). Currie and Carlsen (1985, 1987a) found that the larval lamprey withdraws its head by increasing the curvature of preexisting bends along the length of its body and that bending is related to an amplitude differential in muscle activity between concave and convex sides of body bends with high amplitude on the concave side.

The withdrawal behavior differs in fundamental ways from the C-start behavior, the most common startle response to

head-directed stimuli in fishes (reviewed by Domenici and Blake, 1997). While the withdrawal appears to involve a single stage of muscle activity and movement, the C-start generally includes several stages of movement (Weihs, 1973). The first is a preparatory stage (stage 1) during which the body bends and may turn but with minimal translation of the center of mass. The second is a propulsive stage (stage 2) during which the fish takes its first propulsive tail stroke and the center of mass moves away from the stimulus. Stage 2 may be followed by burst swimming. In contrast to withdrawal behavior, during C-start stage 1, the fish curves to one side of the body along the length of the axis forming a C-shaped bend. In general, the C-start stage 1 movement is thought to involve unilateral muscle activity, although recent studies indicate that bilateral axial muscle activity occurs in some species (Foreman and Eaton, 1993; Westneat et al., 1998; Tytell and Lauder, 2002). Stage 2 includes a wave of contraction on the opposite side of the trunk from the initial bending (e.g. Jayne and Lauder, 1993; Westneat et al., 1998; Hale et al., 2002).

Propulsive startle behaviors may differ fundamentally due to the orientation of the startle stimulus relative to the body. For example, some species respond to tail-directed stimuli with an S-start behavior in which the initial body movement is an S-shaped bend, while head stimuli result in C-start behaviors. The tail-elicited S-start has been demonstrated to result from a qualitatively different pattern of muscle activity than that recorded for head-elicited C-start behaviors in several species (Hale, 2002; Schrieffer and Hale, 2004). Differences in behavior due to stimulus have also been described within a given type of startle. For example, the angle of head movement during stage 1 of the C-start is greater in response to head-directed stimuli than to tail-directed stimuli (Eaton and Emberley, 1991; Liu and Fetcho, 1999).

The main goal of this research is to examine the intraspecific diversity of withdrawal behavior by comparing startles and associated muscle activity patterns of elongate fish in response to head and tail stimuli. By increasing the breadth of startle behaviors and species studied, we aim to provide fundamental data for examination of the neural control and evolution of the startle response. Our specific aims were to determine whether tail stimuli elicited withdrawal responses and, if they did, how they differed from head-elicited startles. We hypothesized that both head and tail stimuli result in withdrawal behaviors but that there will be greater withdrawal of the head to head-directed stimuli and greater withdrawal of the tail to tail-directed stimuli.

A second goal of this work is to provide additional data for broad phylogenetic comparison of withdrawal behaviors. To this end, we chose to work on the species *Erpetoichthys calabaricus*, which preliminary data had shown to perform withdrawal startle responses. *Erpetoichthys* is one of two extant genera, the other being *Polypterus*, of the family Polypteridae. This family is the most basal extant family of actinopterygian fishes and is relatively distant phylogenetically from the other taxa that have been shown to perform withdrawal behaviors. In addition to its interesting phylogenetic position, startle behaviors have been studied in several *Polypterus* species. Both *Polypterus palmas* (Westneat et al., 1998) and *Polypterus senegalus* (Tytell and Lauder, 2002) have been shown to perform C-start startle behaviors. Their startle differs from many teleosts in having marked levels of bilateral muscle activity in stage 1. Based on data showing bilateral activity during withdrawal in larval lamprey (Currie and Carlsen, 1987a) and previous work in polypterids, we hypothesized that muscle is active bilaterally during both head- and tail-elicited startle responses of *E. calabaricus* and that activity will be greater in the direction of bending. To investigate the withdrawal response in *E. calabaricus*, we simultaneously recorded electromyograms (EMGs) at distributed positions on both sides of the body to examine the relationship between body bending and muscle activity. By examining the withdrawal behavior of *E. calabaricus*, we will provide additional data for the broader effort of describing the diversity of startle behaviors within this group.

The neural basis of startle behavior has been studied

extensively for C-start escape behavior (reviewed by Zottoli and Faber, 2000). The response is known to involve the large, paired Mauthner cells, which have somata located in the hindbrain and axons that descend contralaterally the length of the spinal cord. Mauthner neurons have been reported in many diverse fishes and aquatic amphibians (Zottoli, 1978; Stefanelli, 1980). One distinguishing feature of cyprinid M-cells is a unique structure, the so-called axon cap, that surrounds the initial segment of the axon. In the goldfish, fibers that enter the peripheral portion of the axon cap are part of feedforward, feedback and reciprocal inhibitory circuits. While the feedforward network modulates the excitability of the M-cell to sensory stimuli, the reciprocal network between M-cells and the feedback network ensure that only one M-cell fires and that it does so only once (Furukawa and Furshpan, 1963; Faber and Korn, 1978; Faber et al., 1989). Fibers that enter the inner portion of the axon cap have an excitatory influence on the M-cell (Scott et al., 1994).

Withdrawals are also thought to be initiated by Mauthner cell activity. Previous work by Meyers et al. (1998) has demonstrated an association between the morphology of the axon cap and startle behavior. Fishes in which axon caps have not been found include the American eel (*Anguilla rostrata*; Meyers et al., 1998) and lamprey (*Petromyzon marinus*; Rovainen, 1978, 1982; Currie and Carlsen, 1985, 1987a), both of which perform withdrawal behaviors. By contrast, fishes with axon caps, including the elongate lungfish (*Protopterus annectens*; Meyers et al., 1998), perform startles similar to the initial stages of C-start behaviors (Wilson, 1959; Meyers et al., 1998). To further investigate the relationship between the presence of the M-cell axon cap and the escape behavior of elongate fish, we investigate the axon cap structure of *E. calabaricus*. We hypothesized that the M-cell axon cap would be missing in this species.

We found that *E. calabaricus* perform a withdrawal startle response to both head and tail stimuli but that those responses differed with stimulus position. In addition, withdrawal from tail stimuli also acted as the preparatory stage for a second, propulsive stage of movement. Withdrawal behaviors were associated with bilateral muscle activity; however, that activity was quite variable. Surprisingly, Mauthner cells did have an axon cap but the structure appears to be reduced when compared with that of fish that produce C-start behaviors.

## Materials and methods

### Fish

Rope fish (*Erpetoichthys calabaricus* Smith 1865) were obtained from a fish wholesaler in Chicago, IL, USA. The fish were maintained in aquaria at 20°C and fed daily except during the day before an experiment. Animal care and experimentation were approved by the institutional animal care committee at the University of Chicago.

Four fish ranging from 23.6 cm to 26.5 cm total length (25.5±1.3 cm; mean ± s.d.) and 20.1 cm to 22.3 cm standard length were examined. Fish wet masses ranged from 15.6 g to

28.0 g ( $20.2 \pm 6.1$  g); however, because we needed to leave electrodes in place during measurements so that we could later confirm their positions with dissection, the masses measured are not as accurate a measure of size as fish length. Center of mass along the long axis of the body was determined in the freshly euthanized, straight fish immediately after the experiments. To obtain center of mass, fish were positioned lengthwise on a beam balanced on a central fulcrum. When the rostral end and caudal end of the fish balanced, the longitudinal position of the fish over the fulcrum was recorded as the center of mass. Although center of mass will vary as an animal moves, the center of mass measured in the straight position is a common approximation in the kinematics literature and will be used here.

#### Kinematics

For the experimental tank, we used an aquarium measuring  $60 \times 60$  cm with a water depth of approximately 25 cm. In the holding and filming tanks, *E. calabaricus* examined generally remained stationary at the bottom of the tank with their heads slightly raised off the floor. Because of the docile nature of the animals, we were able to position them in the center of the tank prior to recording a startle response. Responses were elicited by pinching the head or tail with metal forceps. Head and tail stimuli were generally alternated with a rest period (~5 min) between trials. All responses observed were withdrawal responses. Fish were not responsive to other, perhaps weaker, stimuli tried, including lateral touch to the head and body and vibration of the tank, and combined stimuli such as tapping the bottom of the tank near the head with a dowel.

High-speed video (250 Hz) of the ventral view of the fish was recorded with a Redlake PCI-2000S digital high-speed video system (San Diego, CA, USA). Twenty-four trials were analyzed, three of each stimulus type for each of four fish. The duration of response was recorded directly from the video. Images were viewed and digitized with NIH Image 1.62. Movements of the tip of the head, the tip of the caudal fin and the position of the center of mass, determined for the specimen when straight, were determined from the images. Head tip and tail tip were determined manually from the images. Center of mass was determined in images in which the fish was curved by measuring in equal segment increments along the midline as described previously (Hale, 1999). This method was also used to determine the rostral midline for analysis of head angle. In addition to kinematics recorded in conjunction with EMGs, control kinematics were obtained prior to the surgery in which we implanted the EMG electrode.

#### Electromyography

Fish were anesthetized with 3-aminobenzoic acid ethyl ester (MS-222; Sigma Chemicals, St Louis, MO, USA). *E. calabaricus* recovered very quickly from anesthesia, probably due to their air breathing abilities, and so were kept in a low dose of MS-222 in a shallow pool of water while electrodes were being implanted. Twelve electrodes were implanted in the fish, six on each side of the body, distributed along its length

(Table 1) as described previously (Westneat et al., 1998). After implantation, the electrode leads were glued together into a cable and the fish was transferred to the filming tank to recover from the anesthesia.

Grass P511 digital amplifiers controlled by Grass software (Grass-Telefactor, West Warwick, RI, USA) on a PC were used to amplify and filter the EMG signal from the 12 electrodes. We used a low-pass filter of 100 Hz and a 30 kHz high-pass filter. The signal was then recorded to computer using LabView 5.0.1 software (National Instruments, Austin, TX, USA) and custom virtual instruments for data collection (written by M. Westneat). A synchronizing signal was also recorded on both a 13th channel on the EMG trace and on the high-speed video so that the two data sets could be synchronized for analysis.

Two of the behavioral trials did not have accompanying EMG recordings due to technical difficulties with the electrodes. Overall, 12 withdrawals to tail stimuli (four fish, three trials each) were analyzed as tail responses and 10 trials (four fish, 2–3 trials each) were examined as head responses. We analyzed muscle activity data by digitizing EMG traces with custom LabView software to determine the duration and amplitude of EMG bursts. Because of variation in noise levels among electrodes, we established a baseline noise level for each channel and used that as a cut-off for determining whether muscle was active as previously described (Westneat et al., 1998). The duration between the synchronizing pulse and the first EMG activity was used to align muscle activity and movement. We compared the activity of muscle, the number of electrodes responding and timing of those responses, as well as the duration, mean burst amplitude and area of EMG activity between stimulus types. Due to possible variation among electrodes, for measurement of burst amplitude we compared not only between stimuli across electrodes but also for each electrode independently.

#### Statistics

We used two-way analyses of variance (JMP; SAS Institute, Trumbull, CT, USA) to analyze the kinematic and EMG

Table 1. *Positions of bilateral electrodes*

Landmark	Position (% BL)	Range (% BL)
Center of mass	$42.5 \pm 1.1$	41.1–43.7
Position 1	$13.3 \pm 0.8$	12.3–14.0
Position 2	$32.4 \pm 1.0$	30.7–33.8
Position 3	$48.1 \pm 3.3$	44.0–52.8
Position 4	$62.3 \pm 1.8$	59.8–64.6
Position 5	$74.0 \pm 2.2$	70.9–77.6
Position 6	$84.1 \pm 2.3$	80.7–86.7

There were two electrodes at each longitudinal position (one in left epaxial muscle and one in right epaxial muscle) per fish. Position values are means  $\pm$  s.d.;  $N=4$  individuals. Total body length (BL) ranged from 23.6 to 26.5 cm (mean  $\pm$  s.d.,  $25.45 \pm 1.3$  cm;  $N=4$ ).

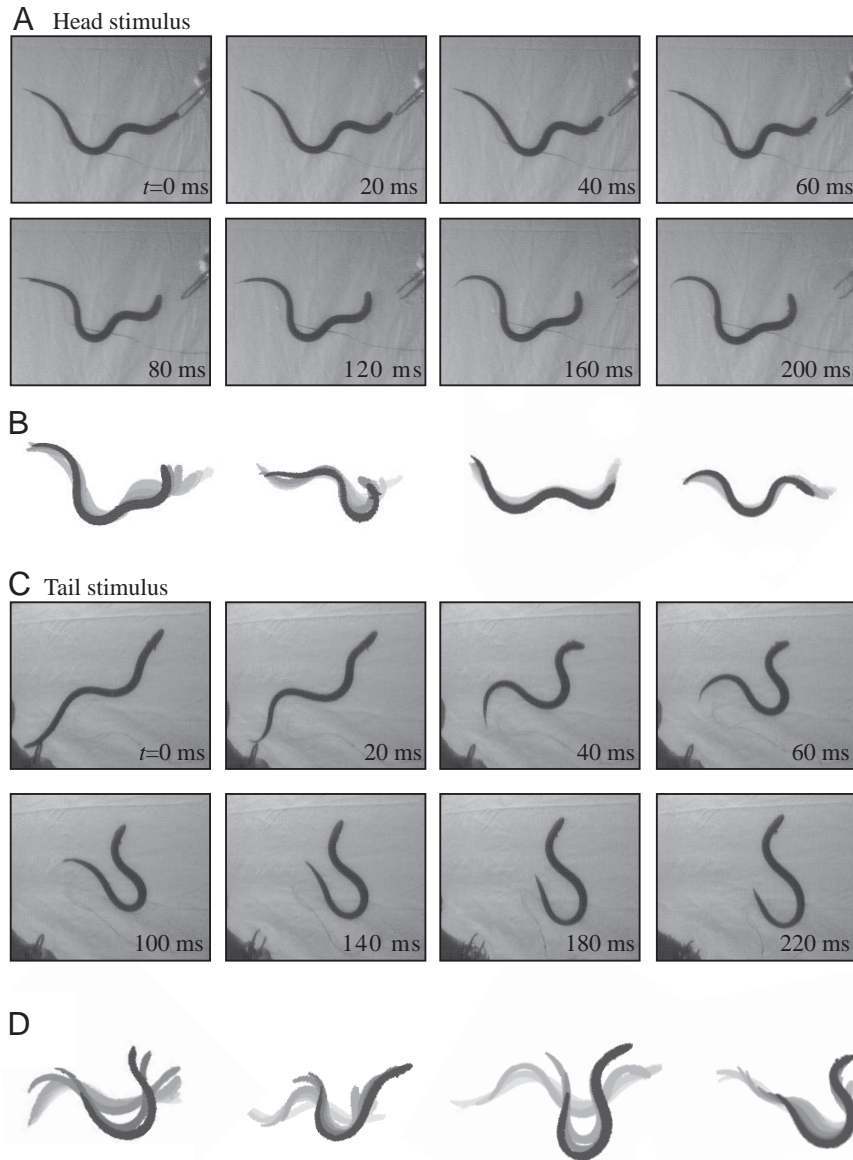


Fig. 1. Images and silhouettes of withdrawal behavior in *Erpetoichthys calabaricus*. All images and silhouettes are oriented with the fish's head to the right. (A) Time series of withdrawal to a head-directed stimulus. The head initially rotates to one side (20 ms) in the pre-transition stage of movement. After the transition point, the head is pulled back from the stimulus and continues to rotate. (B) Silhouettes show examples of four withdrawals to head-directed stimuli. One silhouette is shown for each of the study animals. For an individual withdrawal, the silhouettes get darker through the response. (C) Time series of withdrawal to a tail-directed stimulus. (D) Silhouettes show examples of four withdrawals to tail-directed stimuli. One silhouette is shown for each of the study animals. The response to tail stimulation results in greater movement during withdrawal than the response to head stimulation. In addition, unlike for responses to head stimulation, responses to tail stimulation generally involve a post-withdrawal propulsive stage of movement.

variables to test for differences among individuals and interaction between individual and stimulus type. There was no significant individual effect or interaction term for any of the variables of the withdrawal that differed significantly between responses to head stimuli and responses to tail stimuli. We used a sequential Bonferroni correction (Rice, 1989) to

adjust significance levels for the large number of variables tested. Bonferroni adjustments were made independently for kinematic and electromyographic data sets. For kinematics,  $N=4$  fish, 3 trials per fish per stimulus. For electromyography,  $N=4$  fish, 3 trials per fish to the tail stimulus (12 in total) and 2–3 trials per fish to the head stimulus (10 in total).

### Morphology

We examined the morphology of the Mauthner cells and their axon caps in two *E. calabaricus* brains with a modification of Bodian's silver staining technique (Moulton and Barron, 1967). After kinematic and EMG recording, the experimental fish was euthanized in MS-222. Immediately following euthanization, the head was severed from the body and immersed in 4% paraformaldehyde in  $0.1 \text{ mol l}^{-1}$  phosphate buffer (pH 7.4). The brain was immediately dissected from the skull while in this solution and stored in fresh fixative at  $4^\circ\text{C}$ . Further preparation for paraffin embedding, as well as embedding and sectioning techniques, follows Meyers et al. (1998). Sections were viewed and imaged on a Leica inverted microscope (DM IRB; Wetzlar, Germany) with a Hamamatsu ORCA camera (Hamamatsu City, Japan).

## Results

### Kinematics

Pinching either the head or the tail elicited withdrawal responses in *E. calabaricus* (Fig. 1); however, the strength and pattern of the withdrawal differed markedly between stimulus types and among trials. Fig. 1 illustrates a diversity of withdrawal behaviors: Fig. 1A,B shows responses to head stimuli. Each stack of silhouettes in Fig. 1B is a trial from each of the four fish studied, with the leftmost stack corresponding to the images in Fig. 1A. Fig. 1C,D shows corresponding responses to tail stimuli. Despite the diversity among responses, several kinematic phases of movement emerged that were consistent among the responses to the head-directed stimuli and among the responses to tail-directed stimuli. The first stage of a typical response to a head-directed stimulus is rotation of the head. Head rotation is followed by a second stage of movement in which bending in the trunk and tail retracts the head from its initial position. During this axial bending, the head generally remains in the rotated position. We used the term

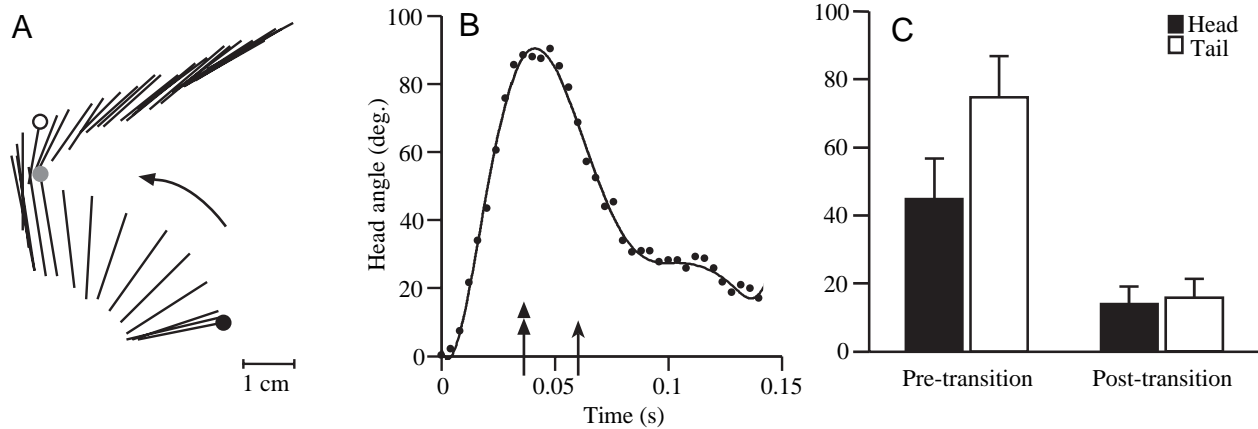


Fig. 2. Head angle rotation during withdrawal responses. (A) Overlay of lines drawn through the stiff rostral section of the fish at 4-ms intervals through the course of a tail stimulus elicited withdrawal. Kinematic landmarks, located at the head, are denoted by black (start of response), grey (transition point) and white (end of withdrawal) circles. (B) Head angle measurements for the same trial as in A, normalized to a start angle of zero and plotted as angle over duration of response. A six-degree polynomial was used to fit the data to a line. In this trial, the transition point (double-headed arrow) is reached at 0.036 s, and the end of withdrawal (single-headed arrow) is at 0.06 s. (C) Comparison of head angle during the periods from initiation of response to the transition point and from the transition point to the end of withdrawal show significantly greater angle movement during the former period ( $P>0.0001$ ) but no statistical difference between head and tail-elicited responses.

Table 2. Kinematic variables in response to head and tail stimulation with F-ratios and P-values for the two-way ANOVA comparison between stimulus types

Variable	Head stimulus	Tail stimulus	F-ratio	P-value
Head angle – start to transition (deg.)	46.1±10.63	75.9±10.9	3.7085	0.0721
Head angle – transition to end (deg.)	15.2±3.9	17.1±4.2	0.1450	0.7084
Head movement (BL)	0.15±0.017	0.22±0.027	4.5952	0.0478
Tail movement (BL)	0.06±0.009	0.35±0.022	150.8221	<b>&lt;0.0001</b>
CM movement (BL)	0.0046±0.0011	0.0062±0.0014	0.6901	0.4184
Initial body extension (%)	81.43±3.24	84.29±2.48	0.5149	0.4834
Min. body extension (%)	66.98±3.12	47.71±2.07	20.4258	<b>0.0003</b>
Duration total (s)	0.195±0.02576	0.095±0.01224	14.4967	<b>0.0015</b>
Duration – start to transition (s)	0.077±0.00804	0.050±0.00937	8.6786	0.0095
Duration – transition to end (s)	0.118±0.02175	0.046±0.007	11.1637	<b>0.0041</b>
Head velocity (BL s <sup>-1</sup> )	1.001±0.235	2.471±0.286	18.1784	<b>0.0006</b>
Tail velocity (BL s <sup>-1</sup> )	0.377±0.079	3.884±0.286	254.9242	<b>&lt;0.0001</b>
CM movement 48 ms post-withdrawal (BL)	0.0312±0.0162	0.14377±0.02983	11.1704	<b>0.0041</b>

Values for kinematic parameters are means ± S.E.M. Sample size is four individuals, three responses to head stimuli and three responses to tail stimuli per fish. P-values in bold are significant after adjustment of table with a sequential Bonferroni correction (Rice, 1989).

'transition point' to identify the time at which the head stops rotating, reaching peak angular deviation from the initial position of the head. We observed only minimal body movement following withdrawal.

The initial response to a tail stimulus is trunk and tail bending in conjunction with head rotation. As both the head and the tail begin to retract, the head undergoes substantial rotation. After the peak rotation of the head, the transition point is reached, and the withdrawal of the head and tail continues. At the end of withdrawal, in most trials the fish were positioned in an omega ( $\Omega$ )-shaped body bend, similar to that observed in some withdrawal trials of the American eel *Anguilla* (Meyers et al., 1998). In others trials, a  $\Omega$  shape was reached shortly thereafter, early in the period of forward movement. In tail-elicited responses, the withdrawal was generally followed by

forward propulsion, including movement through the  $\Omega$ -shaped bend and a caudal tail stroke. Several trials showed a second head rotation during the propulsive stage of movement. To compare post-withdrawal movement between stimuli, we examined performance for 48 ms after the end of the withdrawal. We chose this time interval for several reasons. Since the fish slowly glides to a stop in some trials, we were concerned about accurately determining the absolute end of the movement. In addition, the period up to 48 ms could be measured on all our trials without losing the fish from the field of view.

In order to quantitatively compare the startle behavior between head and tail stimuli, we examined the angle change of the head, movement distance and mean velocity of snout, tail tip and center of mass during the response. The angles of

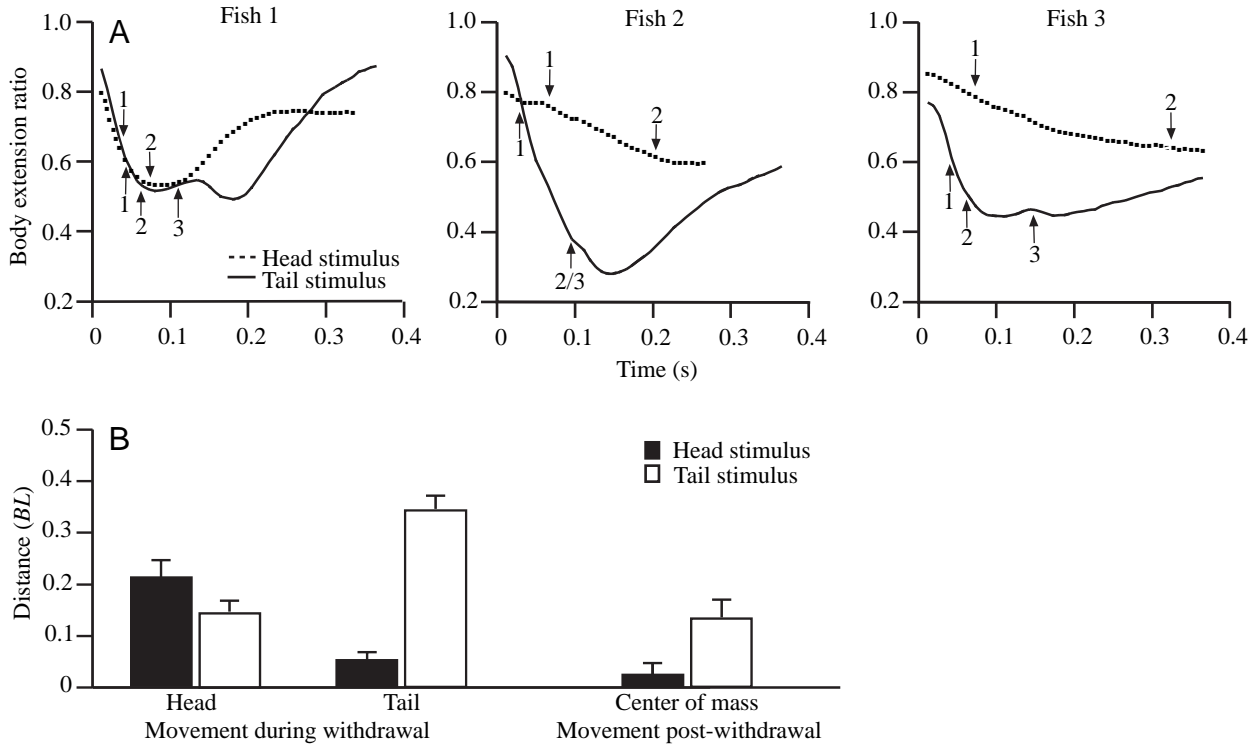


Fig. 3. Body extension ratio and movement during response to head- and tail-directed stimuli. (A) Examples from three different fish showing the change in body extension over the duration of response to head-directed stimuli (dotted line) and tail-directed stimuli (solid line). Start of response was set at 0 ms, and the extension ratio was measured as distance between head and tail divided by total body length (*BL*). Kinematic landmarks are denoted: transition point (1), end of withdrawal (2) and peak of omega-like body shape (3). (B) The overall distance in body lengths moved by the head and the tail during the withdrawal in response to head and tail stimulation. Also shown is the overall distance moved by the center of mass during the 48 ms following the withdrawal (post-withdrawal). There is significantly more post-withdrawal movement of the center of mass in tail-elicited responses than in head-elicited responses.

head rotation were not significantly different between responses to head and tail stimuli for either the period from initiation to the transition point or the period from the transition point to the end of withdrawal (Fig. 2; Table 2). The initial head rotation was  $46.1 \pm 10.6^\circ$  (mean  $\pm$  S.E.M.) for the head-stimulated responses and  $75.9 \pm 10.9^\circ$  for tail-stimulated responses. The amount of rotation achieved from the transition point to the end of withdrawal was significantly lower ( $P < 0.0001$ ) in response to both head and tail stimuli in comparison to the pre-transition movement and was not significantly different between stimuli, with both between 15 and  $20^\circ$  (Table 2).

To test the hypothesis that relative withdrawal of the head and tail is stimulus dependent, we compared the distance of head and tail withdrawal between stimulus types. We found that there was no significant difference in the movement of head and tail in response to head stimulus but that the tail moved significantly further in response to a tail stimulus than a head stimulus (Fig. 3). The difference in tail movement between stimuli was more pronounced, with a nearly sixfold-greater movement in response to tail stimuli than in response to head stimuli ( $P < 0.0001$ ). During withdrawal, the movement of the center of mass was minimal, less than 1% of total body length, and did not differ significantly between stimulus types.

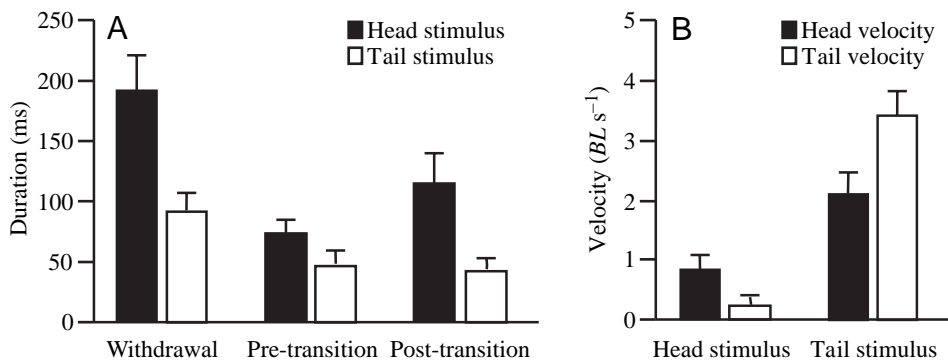
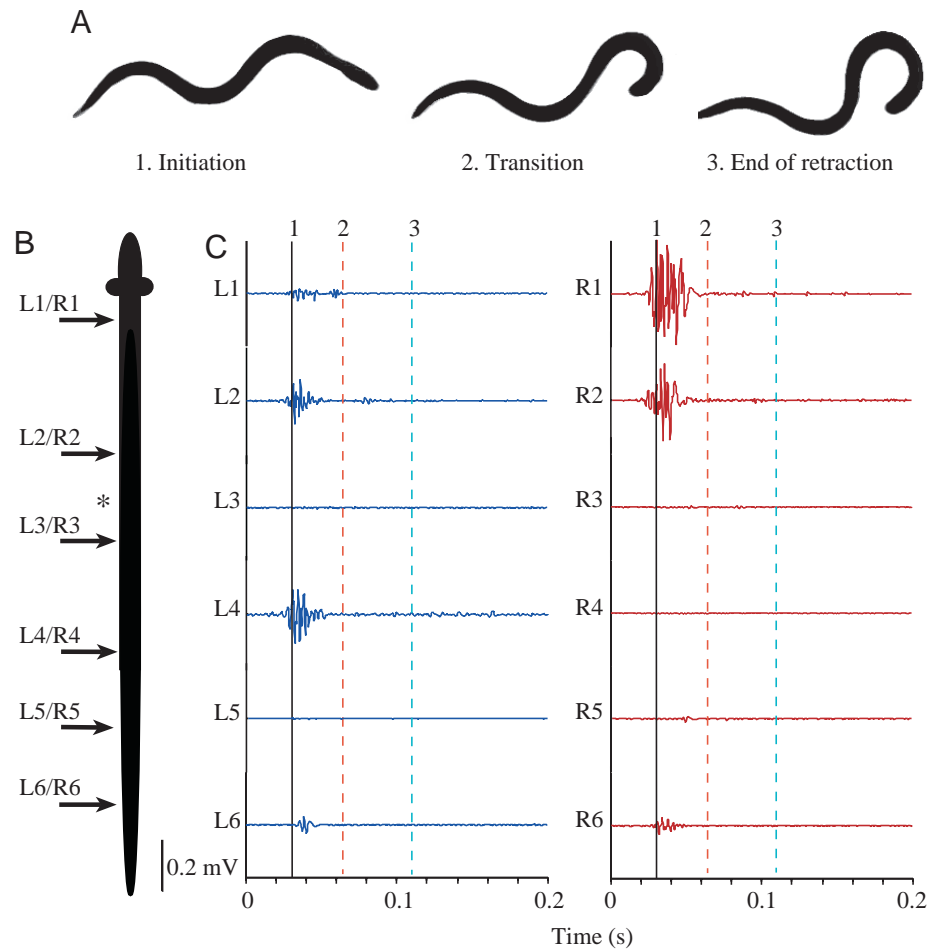


Fig. 4. Duration and velocity of movement during withdrawal response. (A) Bar graphs showing total mean withdrawal duration and mean withdrawal duration before and after transition point (see text description of transition point) for responses elicited by head- and tail-directed stimuli. (B) Mean velocity of movement of the head and tail over the withdrawal response to head- and tail-directed stimuli.

Fig. 5. Example of a withdrawal trial in response to head stimulation. (A) Silhouettes at three kinematic stages – initiation, transition and end of withdrawal – are shown. (B) Six electrodes were implanted in pairs along the length of the body. Asterisk indicates centre of mass. (C) EMG activity for the electrodes shown in B. Broken lines represent kinematic stages illustrated in A and are numbered accordingly: (1) initiation, (2) transition point and (3) end of withdrawal. Compared with tail-stimulation trials (Figs 6, 8) from the same fish, there is relatively little muscle activity in response to head stimulation both in terms of numbers of electrodes active and amplitude of activity. When comparing figures, note that the scale bar for tail stimulation trials is four times that of head stimulation trials, further emphasizing the difference in strength. No muscle activity is present after the initial burst around the onset of kinematic activity. Scale bar, 0.2 mV.



We calculated the extension ratio throughout the response to illustrate the withdrawal of both head and tail during withdrawal behaviors. The extension ratio is the ratio of the distance between head and tail tip to the fish's total length (Fig. 3A). Extension ratio comparisons demonstrate a relatively greater contraction of the body (decrease in extension ratio) in response to tail stimuli than to head stimuli. There was no significant difference in the extension ratio at initiation between stimulus types, both being slightly over 0.8 (Table 2). The extension ratio decreased during both head-elicited and tail-elicited responses. At its minimum (i.e. the closest position of head and tail), the extension ratio was significantly lower ( $P < 0.0003$ ) in response to tail stimuli than to heads (Table 2).

The duration of the total withdrawal was significantly greater in response to head-directed stimuli than to tail-directed stimuli ( $P < 0.002$ ; Fig. 4; Table 2). Withdrawal in response to a head stimulus took an average of 100 ms longer than withdrawal in response to a tail stimulus. For the response to tail-directed stimuli, the total duration was almost evenly split between the duration from initiation to the transition point and from the transition point to the end of withdrawal. A relatively larger portion of the withdrawal occurred during the post-transition period for head-directed responses.

By contrast, mean head velocity and mean tail velocity were significantly greater in response to a tail-directed stimulus than to a head-directed stimulus during the withdrawal ( $P < 0.0014$ ; Table 2). The mean head velocity in response to tail stimulation was over double that of head velocity in response to head stimulation. The difference in tail velocity was more

striking, with the response approximately tenfold higher in response to tail than to head stimuli.

A discrete end point was not identified for post-withdrawal movement. Fish frequently continued moving passively for some time after the withdrawal, often exceeding the limits of recording space and time. When we compared performance during the 48 ms after the end of the withdrawal (Fig. 3), we found significant differences between stimulus types. Examination of post-withdrawal extension ratios shows that the body extends from the maximally contracted position in responses to tail stimuli while this is seldom the case for head stimuli [but see Fig. 3A (Fish 1) for an exception]. We found that, although there was minimal movement of the center of mass of *E. calabaricus* in response to head stimuli, center of mass movement was significantly greater ( $P < 0.005$ ) in response to tail stimuli (Table 2; Fig. 3B).

#### Electromyography

Figs 5, 6 illustrate EMG responses of a single experimental animal to head and tail stimuli with their corresponding behaviors. The response to head stimuli tended to be of low amplitude and involve few electrode positions. The kinematics of the tail response involved propulsive movement after the withdrawal and, associated with those kinematics, more extended EMG activity. Unlike C-starts and S-starts, the EMG

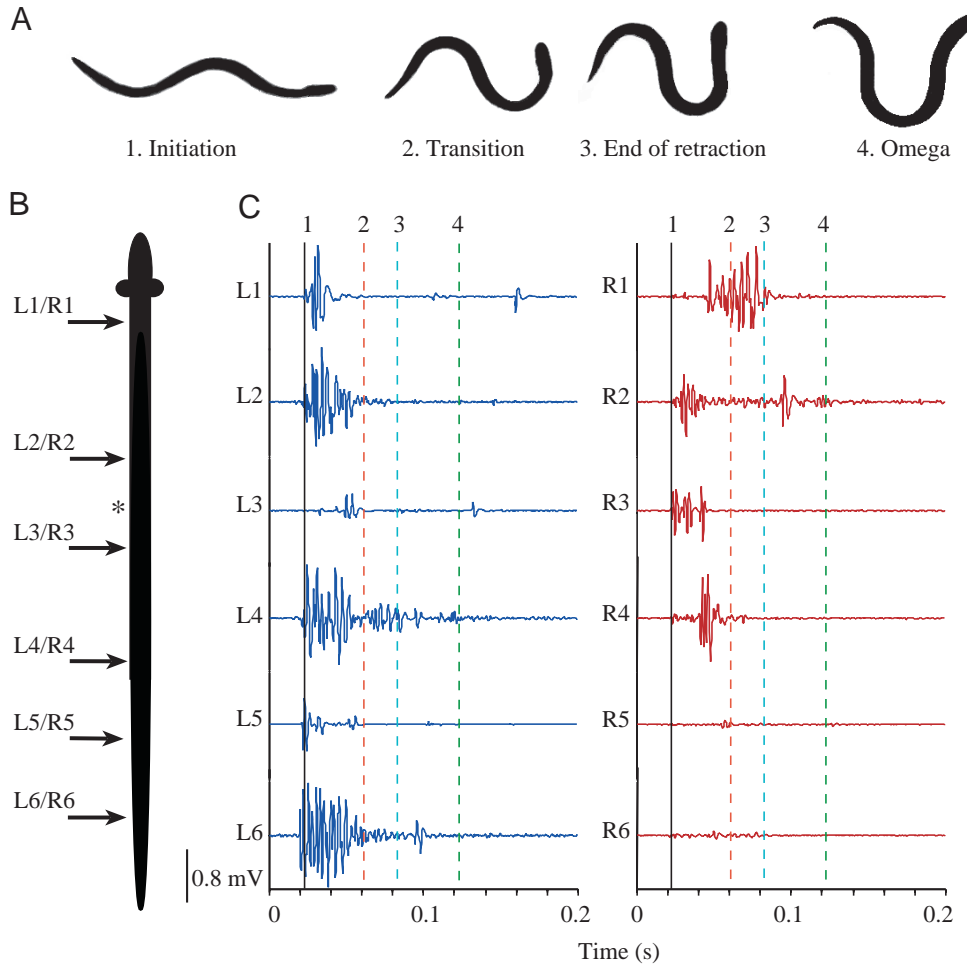


Fig. 6. Example of a withdrawal trial in response to tail stimulation. (A) Silhouettes at four kinematic stages – initiation, transition, end of withdrawal and omega – are shown. (B) Six electrodes were implanted in pairs along the length of the body. Asterisk indicates centre of mass. (C) EMG activity for the electrodes shown in B. Broken lines represent kinematic stages illustrated in A and are numbered accordingly: (1) initiation, (2) transition point, (3) end of withdrawal and (4) omega. Compared with head stimulation trials (Figs 5, 7) from the same fish, there is strong activity of axial muscle in response to tail stimulation. In addition to greater numbers of electrodes being active and those electrodes having stronger activity, there is secondary activity after the initial burst associated with movement after the initial withdrawal. Scale bar, 0.8 mV.

responses to each stimulus type were quite diverse in *E. calabaricus*. To illustrate this diversity, Fig. 7 shows two additional responses to head and tail stimuli for the same individual shown in Figs 5, 6. Comparisons of activity strength among trials were made for individual electrodes within an individual to control for subtle differences in electrode construction or placement. The activity pattern of electrode 6 on the left side of the body illustrates inter-trial variation for responses to both head and tail stimuli. For the responses to head stimulus in Fig. 5, the left side electrode 6 has a weak, delayed response compared with the other electrodes firing on the same side of the body. By contrast, in Fig. 7A, one trial involves a strong early response in that electrode while the other has no response. For the responses to tail stimuli, the left side electrode 6 has a strong early response in the trial in Fig. 6, while in Fig. 7B the first trial shows minimal activity with early onset and the second trial shows stronger activity, but that activity is considerably delayed relative to the first onset of activity.

Despite this variability, we quantified a number of parameters that differed between head and tail responses and we examined the relationship between EMGs and movement patterns (Table 3). For an overall estimate of response strength, we examined the percentage of electrodes responding to the

stimuli and found that a significantly lower percentage responded to head than to tail stimuli ( $P < 0.0001$ ). In response to head stimuli, just over half of the electrodes responded during a withdrawal while all of the electrodes responded to tail stimuli. Of the electrodes active during withdrawals, approximately half as many responded within 5 ms of the first onset of EMG activity in responses to head stimuli than in responses to tail stimuli ( $P < 0.0002$ ; Table 3).

Previous studies (Westneat et al., 1998; Hale et al., 2002) have found interspecific differences in whether muscle activity is unilateral or bilateral during startle behaviors. We examined the proportion of active electrode positions that demonstrated unilateral and bilateral activity for responses to head and tail stimuli by comparing activity in left–right pairs of electrodes. We considered bilateral activity of electrode pairs to have onset times within 5 ms of one another. A significantly greater percentage of electrode positions showed unilateral activity in response to head stimuli than to tail stimuli ( $P < 0.002$ ; Table 3). In responses to both head and tail stimuli, when unilateral activity was observed it was nearly always on the side of the body toward which the fish was bending or in a region with little bending during the response ( $\kappa < 0.01 \text{ cm}^{-1}$ ;  $\kappa$ , a bending index, is the inverse of the radius of curvature);  $93.7 \pm 3.4\%$  of the time for head responses and  $94.5 \pm 5.5\%$  of the time for tail



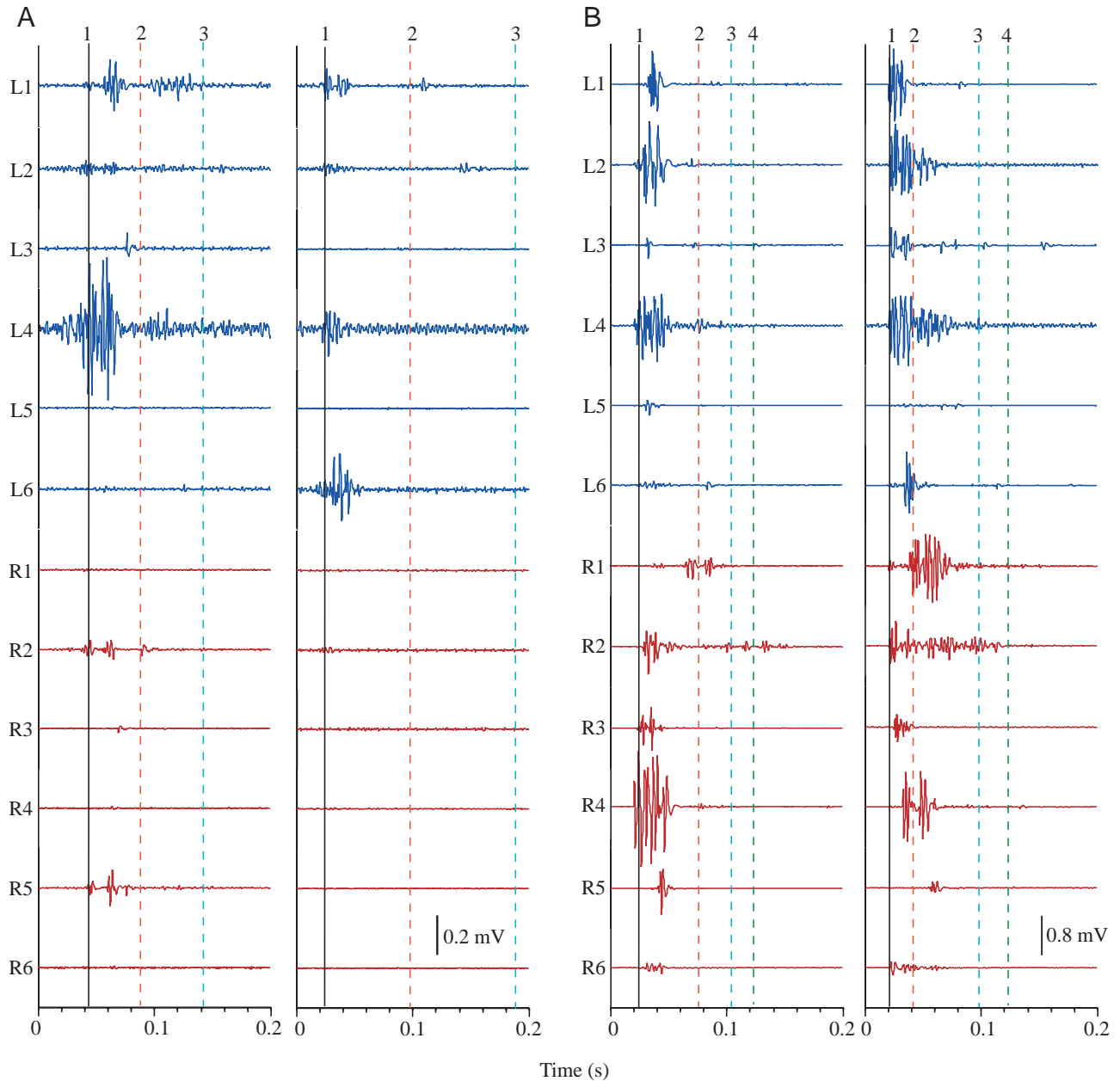


Fig. 7. Additional trials of the fish illustrated in Figs 5, 6 are shown to illustrate the diversity of response within an individual and for the same electrodes. Broken lines and numbers correspond to the numbering in the previous corresponding figures: (1) initiation, (2) transition point, (3) end of withdrawal and (4) omega. Note that the differences in the strengths of activity across electrodes may in part be due to variability in electrode construction and placement. (A) Head stimulation trials comparable to the trial illustrated in Fig. 5. (B) Tail stimulation trials comparable to the trial illustrated in Fig. 6. We show a total of six trials from one fish (three head stimuli and three tail stimuli; Figs 5–7) so that the EMG activity patterns can be compared for individual electrodes across trials. Scale bar: 0.2 mV (head stimulus trials) and 0.8 mV (tail stimulus trials).

responses. There was no significant difference between stimulus types.

The amplitudes of EMGs had similar ranges for both head and tail responses but, on average, were significantly higher ( $P < 0.002$ ) for responses to tail stimuli than for responses to head stimuli. The mean amplitude of head responses was  $0.049 \pm 0.015$  mV (range = 0.004–0.299 mV) while for tail responses it was  $0.112 \pm 0.009$  mV (range = 0.003–0.363 mV) across all active electrodes (Table 3). Because of variation

among electrodes and concerns of combining data from multiple electrodes, we also compared the amplitudes of activity recorded from each of the electrodes independently for each fish to explore this difference. This could only be done for a subset of positions (25 of the total 48) since many were active in one behavior. If an electrode was active in only one of the trials of a particular stimulus we used that number, if it was active in multiple trials we used the mean. In 22 of 25 cases, the amplitude of response to the tail stimulus was greater

Table 3. EMG variables in response to head and tail stimulation with F-ratios and P-values for the two-way ANOVA comparison between stimulus types

Variable	Head stimulus	Tail stimulus	F-ratio	P-value
Electrodes active (%)	56.6±5	100	68.57	<b>&lt;0.0001</b>
Electrodes active within 5 ms of first EMG onset (%)	35.7±7.7	76.7±5.4	20.29	<b>&lt;0.0005</b>
Electrode positions showing unilateral activity (%)	70.0±11.7	22.4±7.4	14.17	<b>&lt;0.002</b>
Of electrodes active unilaterally, % in position in which $\kappa < 0.01$ .	93.7±3.4	94.5±5.5	2.37	0.129
EMG amplitude (mV)	0.049±0.015	0.112±0.009	10.67	<b>&lt;0.002</b>
EMG duration (ms)	20.1±2.2	23.6±1.3	2.02	0.1576
EMG area (mV×ms)	1.06±0.25	2.86±0.31	10.04	<b>&lt;0.002</b>

Values for EMG parameters are means  $\pm$  S.E.M. Sample size is four individuals, 2–3 responses to head stimuli (10 total) and three responses to tail stimuli per fish for variables that examine the percent electrodes responding. For EMG amplitude, duration and area, a total of 146 electrode response were examined, with 39 of those being from responses to head stimuli. See Materials and methods for details of statistics. P-values in bold are significant after adjustment of table with a sequential Bonferroni correction (Rice, 1989).

than that to the head stimulus, and the average difference between the amplitude was  $0.063 \pm 0.019$  mV higher for tail responses than heads.

The duration of EMG activity was not significantly different when compared between head and tail trials (for head, range=4–52 ms, mean= $20.1 \pm 2.2$  ms; for tail, range=4–95 ms, mean= $23.6 \pm 1.3$  ms); however, for this variable there was a significant difference among individuals ( $P < 0.05$ ). EMG area (mV×ms) was quite variable among trials of a given stimulus, and ranges overlapped substantially (for head, range=0.03–7.89 ms×mV, mean= $1.06 \pm 0.25$  ms×mV; for tail, range=0.02–4.88 ms×mV, mean= $2.86 \pm 0.31$  ms×mV) but the means were significantly different between head and tail stimulus trials ( $P < 0.002$ ; Table 3).

#### Morphology

Our preliminary, light microscopy investigation of two *E. calabaricus* brains demonstrates that the species has robust Mauthner cells (Fig. 8). The Mauthner cells are large, with lateral dendrites extending to the root of the eighth cranial nerve. We discovered that an axon cap structure is also present. The axon cap in the goldfish (*Carassius auratus*) can be divided into peripheral and central regions (Bartelmez, 1915; Nakajima and Kohno, 1978). This does not appear to be true

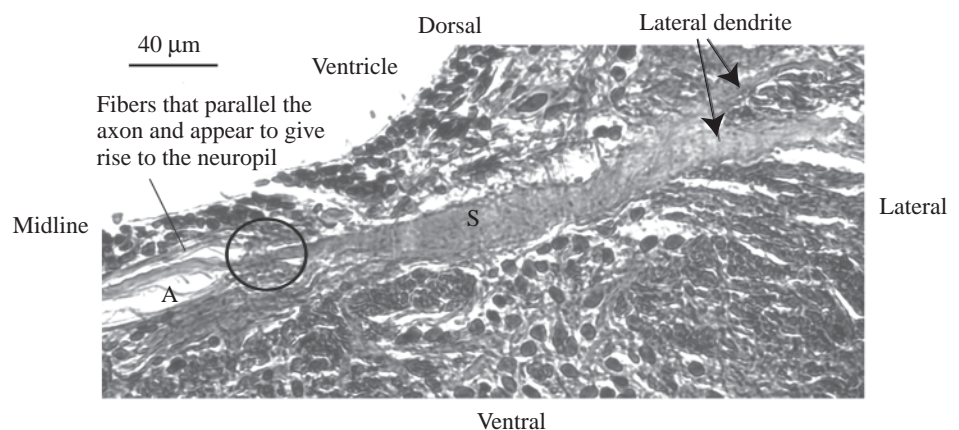
for *E. calabaricus*. Rather, there is a fine network of fibers surrounding the initial segment of the axon.

#### Discussion

Withdrawal is a common startle response to head-directed stimuli for elongate fishes and aquatic amphibians (Ward and Azizi, 2001). In the present study, we demonstrate that *E. calabaricus*, a polypterid fish, also has a withdrawal startle response consisting of an initial head rotation (pre-transition stage) and subsequent withdrawal (post-transition stage). As in other studies (e.g. Eaton et al., 1977; Currie and Carlsen, 1985, 1987a; Currie, 1991; Meyers et al., 1998; Ward and Azizi, 2001), withdrawal to head stimuli was not followed by a propulsive stage of movement, as occurs in most C-start and S-start escape responses.

To investigate intraspecific diversity in withdrawal behaviors, we also examined the startle response of *E. calabaricus* to tail stimuli. We found that *E. calabaricus* performs a withdrawal response when we pinched the tail, supporting our hypothesis that withdrawal occurs in response to both head and tail stimuli. Angles of head movement, common parameters used to describe withdrawal behavior, were not significantly different for either the pre- or the post-

Fig. 8. Cross section (15  $\mu$ m) through the right Mauthner cell of *Erpetoichthys calabaricus*. The soma (S) of the Mauthner cell is centered in this photomicrograph. The lateral dendrite extends towards the right, where it bifurcates (arrows designate two branches) near the entry of the VIIIth cranial nerve. The axon (A) projects to the left toward the midline. Fibers that run parallel to the Mauthner axon project toward the soma and form a network of fibers around the initial segment of the axon to form the axon cap (delineated with a circle). Dorsal is up, and the open space above the Mauthner axon is the IVth ventricle. Scale bar, 40  $\mu$ m.



transition stages of withdrawal, indicating similarity in the pattern of these responses. The most striking difference between withdrawal to head- and tail-stimulus types occurred after the withdrawal event. Unlike the response to head stimuli, responses to tail stimuli involved a post-withdrawal propulsive phase of movement during which the fish moved away from the stimulus. Thus, in the response to tail-directed stimuli, the withdrawal acts both to move the tail rapidly away from the possible threat and as a preparatory stage for propulsion.

In order to compare withdrawal behavior between stimulus types, we analyzed kinematics and muscle activity patterns of the responses. Greater withdrawal of the head occurred in response to head stimuli while greater withdrawal of the tail occurred in response to tail stimuli, supporting our hypothesis that the movement distance is related to the direction of the stimulus. We also observed differences in relative movement between stimulus types. During the response to head-directed stimuli there is minimal movement of the tail (17% of that seen in a tail-elicited response) while during the response to tail stimuli there was considerable movement of the head (68% of that seen in a head-elicited response). In addition to relative movement, other aspects of performance were higher for tail-elicited responses. Overall, tail-elicited responses had shorter durations and higher velocities than did head-elicited responses. The differences between the withdrawal movements may be analogous to differences in the C-start behavior in response to stimulus direction. Stimulus direction has been shown previously to affect the initial bend of the C-start behavior (Eaton and Emberley, 1991; Foreman and Eaton, 1993; Liu and Fetcho, 1999), with longer duration movements of greater head angle occurring in response to head stimulation. By contrast, head angle and pre-transition duration did not differ with stimulus direction for withdrawal in *E. calabaricus*; instead, overall aspects of movement and movement velocity differed. However, although there was not a significant difference in head angle, the mean angle was considerably higher ( $>20^\circ$ ) for responses to the head stimulus than to the tail stimulus, and larger kinematic sample sizes may be able to assess subtle differences that are difficult to pick up due to the high variability among responses.

There are a number of possible reasons for differences in responses to head and tail stimuli. One of them is simply that the tail stimulus was perceived as stronger than the head stimulus. The pinch stimulus was the only stimulus with which we were able to elicit a startle reaction (visual, vibration, touch and auditory stimuli were attempted) and our impression is that it would be difficult to get a stronger head response from these animals.

Another possibility is that, in its role as preparation for a propulsive movement, the body must move more to generate the appropriate response to a tail-directed stimulus than to a head-directed stimulus. Fish that retract tend to be substrate-associated animals and, like the larval lampreys (Currie and Carlsen, 1985), when they withdraw they move their heads from an exposed to a protected environment. A tail stimulus

would not occur in the same context; to move away from the stimulus the animal would have to exit the burrow and swim to another shelter. Little is known of *Erpetoichthys* life history, but we have found no report of burrowing in the species. They do, however, seem to live in structure-rich reedy environments (Greenwood, 1984) and may use this environment in ecologically similar ways to burrowing animals.

Finally, the differences in performance to head and tail stimuli may be due to independent control of subtypes of withdrawal. Recent data (Hale, 2002) demonstrated differences in the muscle activity patterns controlling C-start and S-start responses, elicited, respectively, by head and tail stimuli, that indicate those behaviors are driven by qualitatively different, although likely overlapping, neural circuits. Although we suggest it is more likely that the difference between withdrawals are similar to variations in the C-start response, it is possible that head- and tail-elicited withdrawal behaviors involve fundamentally different neural control, as in the C-start/S-start comparison. Neurophysiological studies are needed to differentiate between the hypotheses.

Our data for *E. calabaricus* add to a growing body of work showing considerable diversity in the kinematics and motor control of startle behaviors (e.g. Foreman and Eaton, 1993; Westneat et al., 1998; Liu and Fetcho, 1999; Hale, 2002; Hale et al., 2002). One aspect of this diversity that has recently received attention is the extent of unilateral activity of axial muscles during initial startle movements. Previous work on goldfish (Foreman and Eaton, 1993) and on *Polypterus* species (Westneat et al., 1998; Tytell and Lauder, 2002) has shown bilateral activity during C-start behavior. In addition, withdrawals in larval lamprey have also been shown to exhibit bilateral muscle activity (Currie and Carlsen, 1985), and other retracting species without Mauthner cell axon caps would be expected to demonstrate the same pattern. Both because of the close relationship of *Erpetoichthys* to *Polypterus* and because *Erpetoichthys* retract, we hypothesized that withdrawal in *E. calabaricus* would also involve bilateral activity. Bilateral muscle activity was frequently but not always present in rope eel withdrawals. However, many electrode pairs showed unilateral activity for both head-elicited responses (70% of electrode pairs) and tail-elicited responses (22% of electrode pairs). The variability in EMG patterns of *E. calabaricus* withdrawals contrasts with the relatively stereotypic patterns of C-start or S-start EMG responses in intraspecific comparisons (e.g. Jayne and Lauder, 1993; Westneat et al., 1998; Hale, 2002).

Although the activity of the Mauthner cell has not been assessed during withdrawal behavior, correlative examination of morphology and startle behavior (Currie and Carlson, 1985; Meyers et al., 1998) suggests that the Mauthner cell is involved in withdrawal and that variation in associated structures, specifically the axon cap, allows for the different forms of the startle behavior. The axon cap of the goldfish is surrounded by glial cells and can be divided into peripheral and central zones (Bartelmez, 1915; Nakajima and Kohno, 1978). The peripheral zone contains M-cell dendrites, and fibers from inhibitory

neurons (i.e. PHP cells), while fibers entering the inner region of the axon cap are excitatory (Scott et al., 1994). *Erpetoichthys* is unique among fishes studied to date in having an axon cap but still performing withdrawal behavior. The axon cap appears to be a simple neuropil with no visible divisions into a peripheral and central zone, as in cyprinids. At the light microscopic level, this 'simple' cap of *Erpetoichthys* appears to be similar to that described in urodeles (Kimmel and Schabtach, 1974; Nakajima and Kohno, 1978) and anurans (Nakajima and Kohno, 1978; Cochran et al., 1980; Cioni et al., 1989).

The lack of an M-cell axon cap has been associated with withdrawal responses in larval lamprey (Currie and Carlson, 1985) and the American eel (Meyers et al., 1998). In the larval lamprey, there is a bilateral activation of M-cells in response to otic capsule stimulation that is thought to occur due to the lack of reciprocal inhibition that is associated with an axon cap (Rovainen, 1967, 1978, 1979; Currie and Carlsen, 1987b). The firing of both M-cells results in bilateral activation of axial musculature (Currie and Carlsen, 1985). *Rana* tadpoles have a reduced M-cell axon cap and lack the recurrent collateral inhibition (i.e. self-inhibition) and reciprocal inhibition (i.e. mutual-inhibition) described for the goldfish. As a result, stimulation of both VIIIth cranial nerves results in simultaneous activation of both M-cells followed by bilateral EMGs. However, stimulation of the contralateral VIIIth nerve resulted in a delayed inhibition that could block activity of the M-cell to ipsilateral VIIIth nerve stimulation (Hackett et al., 1979; Cochran et al., 1980; Rock, 1980). Therefore, it is possible that activation of both M-cells may underlie bilateral EMG responses in *Erpetoichthys*. Further physiological and morphological examination of the Mauthner cells and axon caps in *Erpetoichthys* and the closely related, but C-start-performing, genus *Polypterus* may clarify the roles of both Mauthner cells and axon cap structures and the evolution of withdrawal behavior.

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