By land or by sea: a modified C-start motor pattern drives the terrestrial tail-flip
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ABSTRACT
Aquatic C-start escape responses in teleost fishes are driven by a well-studied network of reticulospinal neurons that produce a motor pattern of simultaneous contraction of axial muscle on the side of the body opposite the threatening stimulus, bending the fish into the characteristic C shape, followed by a traveling wave of muscle contraction on the contralateral side that moves the fish away from the threat. Superficially, the kinematics of the terrestrial tail-flip resemble the C-start, with the anterior body rolling up and over the tail into a tight C shape, followed by straightening as the fish launches off of the caudal peduncle into ballistic flight. We asked whether similar motor control is used for both behaviors in the amphibious mangrove rivulus, Kryptolebias marmoratus. Fine-wire bipolar electrodes were percutaneously inserted into repeatable paired axial locations in five individual fish. Electromyograms synchronized with high-speed video were made of aquatic C-starts, immediately followed by terrestrial tail-flips. Tail-flips took longer to complete than aquatic escapes; correspondingly, muscles were activated for longer durations on land. In the tail-flip, activity was seen in contralateral posterior axial muscle for an extended period of time during the formation of the C shape, likely to press the caudal peduncle against the ground in preparation for launch. Tail-flips thus appear to be produced by modification of the motor pattern driving the aquatic C-start, with differences consistent with the additional requirement of overcoming gravity.

KEY WORDS: Mangrove rivulus, Electromyography, Escape response, Tail-flip, Axial musculature, Motor pattern

INTRODUCTION
The mangrove rivulus, Kryptolebias marmoratus Poey 1880 (Cyprinodontiformes), is an amphibious fish that makes temporary excursions onto land for various reasons: actively pursuing prey at the water–land interface, purposefully leaving the water as a result of poor conditions such as high levels of hydrogen sulfide or anoxia, being stranded at low tide or escaping an aquatic predator (Abel et al., 1987; Regan et al., 2011; Pronko et al., 2013). Minnows, gobies, sculpins and some other groups of teleost fishes are also capable of finding themselves on land through active and passive means (Gibb et al., 2013). Once there, however, these fish must contend with the demands of the terrestrial environment: buoyant support is lacking, gravity must be overcome and new predators such as birds or snakes may be encountered. The mangrove rivulus and many other small fishes lacking modified fins for terrestrial support and locomotion move by means of a ‘tail-flip’, a coordinated movement in which the fish, lying on its side, lifts the head and rolls it over the tail, then straightens the body, finally launching off of the tail to become a ballistic projectile (Gibb et al., 2011, 2013). An unanswered question is how the tail-flip behavior is generated.

The aquatic C-start escape response, a fast start regulated by the Mauthner cell and its associated network of escape neurons, has been thoroughly studied across the fish phylogeny (Weihs, 1973; Eaton et al., 1977; Eaton and Hackett, 1984; Domenici and Blake, 1997; Hale et al., 2002). This characteristic escape response consists of Stage 1, where a fish contracts its muscles to curve its body away from the stimulus, followed by Stage 2, where the fish propels its body out of the C shape and swims away. In water, mangrove rivulus perform typical C-starts, although they are considerably slower (present study) compared with C-starts of similarly sized fishes (Webb, 1978a; Domenici and Blake, 1993); on land, the tail-flip behavior, which is performed by the mangrove rivulus, looks superficially similar to the C-start escape response (Fig. 1) (Gibb et al., 2011, 2013). The mangrove rivulus eliciting the terrestrial tail-flip show similar axial movements in what appears to be a stereotyped behavior (Wainwright et al., 2011).

The neuromuscular control of specific behaviors might vary as a result of the physical environment in which the behavior occurs. Fishes, salamanders and anurans use the same appendages to swim, walk, trot or jump in water and on land, and the neural control driving the muscular output may be different in water and land because of the viscosity of the medium, overcoming gravitational forces on land compared with buoyancy in water, and the ability to generate thrust (Gillis, 2000; Gillis and Biewener, 2000; Ellerby et al., 2001; Gillis and Blob, 2001; Horner and Jayne, 2008; Rivera and Blob, 2010). The ‘collective output’ (Nishikawa et al., 2007) might be masked because of the physical properties of the medium. We therefore asked whether the motor pattern used to generate the aquatic escape response is conserved or whether a different motor pattern exists for the terrestrial tail-flip behavior. We also wanted to explore whether these respective motor patterns might be considered stereotyped or whether there is a range of flexibility that leads to similar or different kinetic responses in both media. Specifically, we hypothesize that (1) the motor patterns will be grossly similar between the two media because of very similar escape kinematics, and (2) the muscle intensity and duration to complete the escapes will be greater and longer on land, respectively, because of the need to overcome gravitational forces.

MATERIALS AND METHODS
Five mangrove rivulus (K. marmoratus) were caught on Long Caye, Lighthouse Reef Atoll, Belize, Central America (17°13′04.9″N, 87°35′38.7″W), in August 2011 using the cup trap method (Taylor, 1990) and transferred to Wake Forest University under the Belize Agricultural Health Authority (certificate no. BAHA-IADC 11.08-L009 to B.M.P.). Fish were maintained for 2 years individually in 32 ounce (0.95 liter) containers with 25 ppt seawater and fed 2 ml...
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position; 0.5 mm of the wire was still coated in Teflon to prevent any
the tip of the bi-polar electrodes. The now-exposed bi-polar wires
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(Fig. 2). All electrodes were implanted through the barrel of the
one electrode embedded into the body cavity to act as a ground
muscles on the posterior part of the body (left and right sides), with
length, was implanted in the anterior epaxial musculature of the
using 30 gauge hypodermic needles. One pair of electrodes, 2 m in
pentacle (Fig. 2) was 3.29±0.07 cm (mean±s.d.), and the total
length (TL), measured from the tip of the snout to the end of the
tail, was 4.05±0.05 cm. The average mass of these fish was
0.636±0.037 g. To our knowledge, these are the smallest vertebrates
used to conduct electromyographic studies for aquatic and terrestrial
kinematics. All procedures were approved by the Wake Forest
University IACUC (protocols A11-134 and A14-078).

**Surgical methods**

Individual fish were anesthetized by immersion in MS-222 (tricaine
methanesulfonate; 0.1 g MS-222 per liter of brackish water) with
one pellet of potassium hydroxide to neutralize the acidic MS-222.
Once the anesthetic took effect (15 to 20 min), which was indicated
by the fish no longer being able to maintain its upright body
orientation, we inserted a 6-0 suture via a needle and tied two
surgeon’s knots to secure the suture to the mid-dorsal area of the fish.
Five bipolar fine-wire electrodes (50 µm wire dia.; California Fine
Wire Co., Grover Beach, CA, USA) were percutaneously inserted
into repeatable locations along the axial musculature of each fish
using 30 gauge hypodermic needles. One pair of electrodes, 2 m in
length, was implanted in the anterior epaxial musculature of the
body (left and right sides) and one pair in the lateralis superficialis
muscles on the posterior part of the body (left and right sides), with
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(Fig. 2). All electrodes were implanted through the barrel of the
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the tip of the bi-polar electrodes. The now-exposed bi-polar wires
were split from the tip of the electrode to 1.0 mm down their long
axis and bent back over the bevel of the needle into a hooked
position; 0.5 mm of the wire was still coated in Teflon to prevent any
noise between the two wires. Once all the electrodes were implanted,
which took approximately 10–12 min in total surgical duration, we
 glued all of the electrodes together with rubber cement to form a
single flexible cable, and tied a double surgeon’s knot using the 6-0
suture, which we previously attached to the body, to secure the cable
against the body. We positioned the cable away from the paired and
median fins by looping it on top of the body, then kinking it to rest
perpendicular to the long axis of the body in the vertical direction.

**Artemia spp. nauplii** daily. The animal facility was temperature-
controlled (25°C) with a 12 h:12 h light:dark cycle. The standard
length (SL), measured from the tip of the snout to the caudal
peduncle (Fig. 2) was 3.29±0.07 cm (mean±s.d.), and the total
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perpendicular to the long axis of the body in the vertical direction.

Upon completion of implanting the electrodes, a fish was placed
into a 32 ounce recovery tank (no anesthetic present; 25 ppt seawater)
as we transferred the fish from the surgery platform into a 10 gallon
(37.9 liter) tank (25 ppt seawater) to conduct the electromyography
(EMG) trials. Specimens were allowed to recover for a minimum of
20 min after the opening and closing of their opercles returned to a
normal pattern. The recovery time followed the general rule about
being twice as long as the surgical procedure (Flammang and Launder,
2008). We connected the electrodes to separate channels of differential
AC amplifiers (AM Systems, Sequim, WA, USA), with a gain of
10,000×, a 60 Hz notch filter and a bandwidth of 300–10,000 Hz, and
recorded the EMG signals on a Lenovo ThinkPad computer (Lenovo
Systems, Morrisville, NC, USA). Electromyograms were captured at
3230 Hz and analyzed in AcqKnowledge version 3.9.1 (BIOPAC
Systems, Goleta, CA, USA). Onset and offset times of muscle activity
in the electromyogram were determined by eye, using the criterion that
the burst activity was at least twice the amplitude of the baseline noise
level (usually extremely small). All cables and the tank were covered
in a Faraday cage (aluminum mesh; grounded) to reduce potential
electrical noise.

We used a high-speed video camera (Fastec Imaging, San Diego,
CA, USA) positioned in a lateral view to record the aquatic and
terrestrial escape responses of each fish (500 frames s⁻¹). Two LED
light arrays (WF Studio Systems, Cowboy Studio, Allen, TX, USA)
were used for illumination. A trigger pulse stopped the video
recording and was also captured in AcqKnowledge as a separate
channel; this pulse was used to synchronize the video with EMG
signals. All videos were imported into ImageJ (National Institutes of
Health, Bethesda, MD, USA) (Rasband, 2008) for kinematic
analysis.

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Fig. 1. Diagram of the aquatic C-start escape response, modified from
Hale and colleagues (2002), depicting the various stages of the behavior.
Grossly similar is the terrestrial tail-flip, exhibiting similar body modification
for both Stage 1 (maximum body curvature) and Stage 2 (straightening the body
as it propels out of the C-bend).

**Fig. 2. Diagram of implanted locations of the bi-polar fine-wire electrodes
along the body.** (A) Dorsal view of the mangrove rivulus with landmarks
(oranges circles) of the four fine-wire electrodes implanted into repeatable
locations on the body. A fifth electrode was implanted into the body cavity to act
as a ground (not shown). The circular-profiled, lobed tail is illustrated to
demonstrate its plate-like form when it lies flush with the substrate due to the
torquing of the posterior region of the body when the fish is out of the water.
(B) The fish is in a C-shape position at the end of Stage 1 during the escape
response, with the concave side defined as the ipsilateral side (shown in white)
for a given escape response, and the convex side defined as the contralateral
side (shown in yellow). Standard length (SL) is the distance from the tip of the
snout to the caudal peduncle when the longitudinal axis of the fish’s body is
completely straight. Chord length (CL) is the distance from the tip of the snout
to the caudal peduncle, regardless of body position.
We approached the head of the fish with the handle end of a dip net to elicit an aquatic escape response (N=1–4 trials per specimen; N=5 specimens). Immediately following the aquatic trials, individual specimens were placed onto wetted bench liner paper adjacent to the aquatic tank and the end of a dip net or an index finger was used to elicit a terrestrial escape response (N=1–4 trials per specimen). No differences in the fish’s reaction time were observed between the two stimuli. The same individuals were used for both the aquatic and terrestrial trials. Aquatic and terrestrial kinematics were recorded before implantation of electrodes as a control to measure whether escape responses differed with and without the electrodes. Kinematic measurements included time to reach maximum body curvature (Stage 1 duration), curvature coefficient and entire duration of either the C-start escape response or the terrestrial tail-flip. No statistical differences were observed in the same individuals when responses with and without electrodes were compared (Student’s t-test: P>0.05; SPSS v. 19.0, Armonk, NY, USA).

Upon completion of the EMG recordings, we euthanized each fish via an overdose of MS-222 and dissected the electrodes to verify they were implanted in the targeted muscle. Distance measurements were taken from the tip of the snout to a specific vertebra for each electrode. We performed a two-tailed Student’s t-test to note whether the location of the paired electrodes (anterior and posterior pairs) along the axial musculature was offset (left versus right). We found no differences between the left and right sides of the body where we implanted the anterior and posterior pairs of electrodes, respectively (paired anterior muscles: P=0.780; paired posterior muscles: P=0.075). For example, if we recorded asynchronous muscle activity on the left and right sides of the body in the anterior region, we would know the difference in onset time of muscle activation was not due to the electrodes being placed at different positions along the length of the body as the activation propagates along the long axis of the body.

To compare motor patterns of aquatic versus terrestrial escape responses, we performed a multivariate ANOVA [MANOVA; fixed factor=medium (water versus land)] using the following variables: muscle onset time (ms), muscle duration (ms) and the rectified integrated area of the muscle burst (a measure of muscle activation intensity; V×ms). We also compared the peak voltage (V) generated by all four muscles during the aquatic escape response and the terrestrial tail-flip using a paired two-sample t-test. To compare the kinematics of the aquatic and terrestrial escape responses, we measured the duration to complete Stage 1 (when the body reached its maximum curvature) and Stage 2 (when the fish propelled its body out of the C-bend and became straight in the water, and when the fish launched off the terrestrial substrate to become a ballistic projectile). To standardize our comparisons of the maximum body curvature reached at the end of Stage 1 and the duration to complete Stage 1 in both media, we calculated the curvature coefficient (Webb, 1978b; Brainerd and Patek, 1998). We slightly modified this dimensionless number by measuring the chord length (CL), which we described as the distance from the tip of the snout to the caudal peduncle when the fish was bent, divided by the SL of the body when the fish was straight (tip of the snout to the caudal peduncle; Fig. 2). Brainerd and Patek (1998) measured the CL (when fish was bent) and SL (when fish was straight) from the base of the head to the caudal peduncle. Because body curvature varied among individuals within and across media, we recorded the duration to reach a curvature coefficient of 0.74, which was achieved by all specimens, to observe whether there were differences in reaching the same body curvature in different media. We used a MANOVA to compare our results and a Tukey’s post hoc test to determine where actual differences occurred. For all statistics used, we set α=0.05 to determine significant differences.

RESULTS
We observed differences in the motor pattern of the aquatic escape response versus the terrestrial tail-flip in the mangrove rivulus (MANOVA with media as the fixed factor: Φș,6ο=53.837, P<0.0001, Wilk’s lambda=0.182, η²=0.818). In the aquatic escape response, there was initially synchronous activity in the anterior and posterior axial muscle on the side of the fish away from the stimulus [ipsilateral’ (to the C-bend) muscles; Tukey’s post hoc test on muscle onset: P=0.999], where the fish reached its maximum bending in the form of a C shape at the end of Stage 1 (Figs 3, 4). A traveling wave (anterior to posterior) of asynchronous activity followed in the contralateral axial muscle (Tukey’s post hoc test on muscle onset: P<0.001), which allowed the fish to propel itself out
of the C shape and away from the stimulus, thus completing Stage 2 of the escape response (Fig. 4, see Movie 1).

The terrestrial tail-flip was driven by a motor pattern similar to that of the aquatic C-start, but with consistent differences: the ipsilateral (concave side of the C) muscles showed asynchronous activity (anterior activated before the posterior; Tukey’s post hoc test: \( P<0.001 \)), whereas the contralateral muscles showed synchronous activity after a short delay following ipsilateral muscle activity (Tukey’s post hoc test on contralateral muscle onset: \( P=0.993 \); Fig. 4, see Movie 2). Stage 1 occupied a shorter duration in water (26.6±1.5 ms; mean±s.e.m.) compared with terrestrial trials (80.0±2.9 ms; MANOVA: \( P<0.0001 \)). Stage 2 was also significantly shorter in duration aquatically (30.5±2.1 ms) compared with Stage 2 on land (42.9±1.5 ms; MANOVA: \( P<0.0001 \)). Overall, the motor pattern of the aquatic escape response was similar to that of other adult teleosts and fit within the parameters of being a C-start escape response (≤100 ms) (Jayne and Lauder, 1993).

Note that the average aquatic escape response occurred in 57.1±3.1 ms, whereas the average terrestrial tail-flip took 122.9±3.6 ms, well beyond the duration of being considered a fast-start escape response.

In the tail-flip, the onset time for muscle activation occurred earlier relative to time zero (first movement of the fish’s body) than in the aquatic escape response (MANOVA: \( d.f.=7, F=8.508, P=0.005 \); Fig. 4). Duration of total muscle activation was longer for the terrestrial escape compared with the aquatic C-start (\( P<0.002 \)). Muscle intensity (V×ms) was greater during the terrestrial tail-flip compared with the aquatic escape response (\( P<0.004 \); Fig. 4). Peak voltage generated by the four muscles of the mangrove rivulus in the aquatic escape response (0.56±0.159 V, mean±s.e.m.) was not different compared with the peak voltage generated by the muscles during the terrestrial tail-flip (0.61±0.125 V; paired two-sample \( t \)-test: \( P>0.808 \)). To remove duration of muscle activation as a confounding factor in calculating muscle intensity across the two media, we divided the intensity (V×ms) of the muscle burst by its duration (ms) to obtain an average voltage value. The mean (±s.d.) intensity value in water was 0.27±0.15 V, and on land it was 0.23±0.07 V. No statistical difference was observed (paired two-sample \( t \)-test: \( P=0.34 \)).

When comparing the maximum body curvature achieved, there was a significant difference between the aquatic and terrestrial curvature coefficient (Student’s \( t \)-test assuming unequal variance: \( P<0.002 \)). Specimens on land achieved a greater amount of body curvature (C shape) compared with the curvature generated during the aquatic escape response. When comparing the duration to reach a curvature coefficient of 0.74, fish reached that curvature coefficient faster in water compared with specimens on land (Student’s \( t \)-test assuming equal variance: \( P<0.0001 \)).

When comparing the total duration of the escape behaviors, mangrove rivulus showed a lower coefficient of variation (standard deviation divided by the mean) (Wainwright et al., 2011) in the terrestrial tail-flip (0.248) compared with the aquatic escape response (0.490). The tail-flip appears to be a more stereotyped behavior when eliciting a response via the same stimulus compared with a much more variable escape response in the water under the same conditions other than the medium.

**DISCUSSION**

We conclude that a modification of the motor pattern producing the aquatic C-start behavior drives the terrestrial tail-flip. The two behaviors are similar in the overall kinematic pattern of bending the body into a C shape, followed by straightening and movement away from the starting position. The motor patterns are similar in the periods of overlapping activity in the anterior and posterior axial muscle, first on the ipsilateral (concave) side and then on the contralateral side. However, we also note the following differences in the tail-flip compared with the aquatic escape response: (1) the earlier, asynchronous activation of anterior and posterior ipsilateral muscle in advance of Stage 1; (2) the synchronous onset of activity in the contralateral axial muscle during Stage 1 (never seen in the aquatic C-start for the mangrove rivulus); and (3) the longer duration of muscle activation (both ipsi- and contralateral). We hypothesize that during Stage 1 of the tail-flip, the asynchronous ipsilateral muscle bursts reflect the anterior-to-posterior pattern of ‘peeling’ the body away from the substrate, where the fish was likely to press the caudal peduncle against the substrate in preparation for the launch to become a ballistic projectile via early offset of the ipsilateral anterior muscle with longer activity (delayed offset) in the ipsilateral posterior muscle. The overlap in muscle activity by the anterior and posterior muscles contralateral to the C shape powered the fish’s body through Stage 1 and may pressurize the body and store elastic energy. Activity of the anterior muscle might control the bending so the behavior is smoothly executed. The earlier onset time (relative to time zero) and longer duration of ipsilateral muscle activity is likely required to overcome gravitational loads when bending into the C shape or to store the elastic elements for energy storage.

It is possible that more than muscle activity alone may be responsible for the rapid launch behavior. Pre-loading of the contralateral axial muscle during the initial bend into the C shape (suggested by the low-level activity during Stage 1) would stretch the series and parallel elastic elements of the muscle, as well as the connective tissue of the myosepta and superficial collagen fibers.
(Wainwright, 1983), storing elastic strain energy. Additionally, bending of the thin, splint-like neural and hemal spines that span multiple intervertebral joints (Ashley-Ross et al., 2014) may occur as a result of body bending (Videler, 1993), potentially storing strain energy as well. Return of this energy may hasten straightening of the body axis and the consequent ballistic launch. Finally, many cyprinodontiform fishes that make terrestrial forays onto land share a similar morphology of fused hypurals (Parenti, 1981). Mangrove rivulus have fused hypural bones, which appears to be characteristic of fishes capable of performing coordinated tail-flips (Gibb et al., 2013; Ashley-Ross et al., 2014). It is still unclear whether the fused hypurals bend (and thus store elastic strain energy) during the tail-flip, or whether they serve primarily to provide a more stable platform from which to control the launch.

What ultimately underlies the difference between aquatic and terrestrial escape behaviors? Environmental variables may combine with motor pattern plasticity to generate distinct behaviors; gravitational loads, buoyancy, viscosity and other environmental mechanics may lead to performance differences, or multifunctionality, in water and land (Nishikawa et al., 2007; Denny, 1993; Vogel, 1994). Many animals are capable of multifunctionality, such as turtles using their appendages to paddle and walk (Earhart and Stein, 2000), eels undulating their bodies for swimming in water and moving onto land (Gillis, 1998, 2000; Biewener and Gillis, 1999; Ellery et al., 2001), the Pacific leaping blenny using its median and paired fins to swim in water and for hopping and twisting on land (Hsieh, 2010), and the mangrove rivulus using its body for transitioning onto land (Pronko et al., 2013) and tail-flipping once out of the water (Gibb et al., 2013; Ashley-Ross et al., 2014). Nishikawa and colleagues (2007) hypothesized that the environment may be an important factor in causing neural circuits to reorganize, making it difficult to tease apart the ‘collective output’ involving the relationships between the brain, sensory organs, muscles and the environment in terms of the evolution of a novel muscle pattern or behavior. Rivera and Blob (2010) raised similar questions involving differences found in muscle duration and intensity for the slider turtle (Trachemys scripta) when swimming or walking on land, where the same forelimb and hindlimb muscles were activated to generate both behaviors across the two environments. In the toad (Bufo marinus), Gillis and Biewener (2000) found differences in the mechanical behavior of the hindlimbs between jumping and swimming, which they interpreted as being due to a change in the motor output that may, in turn, have been influenced by differences in the physical environment. In our study, we observed the same muscles being recruited for both aquatic and terrestrial escapes, with differences found in the motor patterns generated that ultimately provided similar kinematic output. Horner and Jayne (2014) showed that lungfish increase their lateral bending of the vertebral column and increase the amplitude of axial muscle activity when moving on land. There was also an increase in the duration of muscle activity and the axial bending through a locomotor cycle, similar to the increased time of muscle activity and the kinematic output for mangrove rivulus when moving on land. Although lungfish do not tail-flip, the motor patterns show similar responses to the change in environment.

In the aquatic environment, by changing the approach angle and location of the stimulus to the fish of interest, the escape elicited will vary considerably, demonstrating the plasticity of the fast-start escape response generated by the Mauthner cells and their associated network of brainstem escape neurons (Eaton et al., 2001; Foreman and Eaton, 1993). This ‘direction change concept’ allows for the differences in both agonist and antagonist muscle activity, with respect to the side of the stimulus, and may be responsible for why we observed statistically different motor patterns in the escape response in mangrove rivulus across both aquatic and terrestrial media. We observed a greater amount of stereotypy (lower coefficient of variation) in the tail-flip compared with that of the aquatic C-start. This might have been due to the fish having a reduced number of possible directions in which to go in a successful tail-flip (which are always toward the tail and away from the substrate), as opposed to the aquatic C-start, where the fish could escape in nearly any direction. In both water and on land, we approached the fish with a stimulus from a 45 deg angle toward the head region. This angle was kept constant throughout the trials; however, the stimulus was always directed anteriorly when on land and sometimes anteriorly and off to one side of the body in the aquatic trials, which may have been enough to generate a different response as a result of the Mauthner neurons responding differently to changes in stimulus angle, and thus a difference in muscle burst onset and offset to power through Stages 1 and 2 of the escape response. Even so, the ‘direction change’ concept does not account for all of the observed differences between aquatic and terrestrial escapes, most notably the different finding of extended contralateral muscle activity during Stage 1. Thus, the terrestrial tail-flip is not merely a C-start performed, unchanged, on land, but an example of how an effective behavior for a new environment may be produced by active modification of an ancient, robust, and flexible motor pattern driving rapid movements in fishes.
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