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A Method For Detailed Movement Pattern Analysis Of Tadpole Startle Response

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Abstract

Prolonged space flight, specifically microgravity, presents a problem for space exploration. Animal models with altered connections of the vestibular ear, and thus altered gravity sensation, would allow the examination of the effects of microgravity and how various countermeasures can establish normal function. We describe an experimental apparatus to monitor the effects of ear manipulations to generate asymmetric gravity input on the tadpole escape response. To perform the movement pattern analysis, an imaging apparatus was developed that uses a high-speed camera to obtain time-resolved, high-resolution images of tadpole movements. Movements were recorded in a temperature-controlled test chamber following mechanical stimulation with a solenoid actuator, to elicit a C-start response. Temperature within the test cell was controlled with a recirculating water bath. *Xenopus laevis* embryos were obtained using a standard fertilization technique. Tadpole response to a controlled perturbation was recorded in unprecedented detail and the approach was validated by describing the distinct differences in response between normal and one-eared tadpoles. The experimental apparatus and methods form an important element of a rigorous investigation into the response of the tadpole vestibular system to mechanical and biochemical manipulations, and can ultimately contribute to improved understanding of the effects of altered gravity perception on humans.

Keywords

microgravity; vestibular alterations; C-start response; startle response; *Xenopus laevis* embryos; high-speed imaging; movement pattern analysis

Gravity is the only constant sensory stimulus to which life has been exposed that cannot be manipulated except through exposure to hyper- or microgravity (Ronca, Fritsch, Bruce, &

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Ethics Statement: All methods were performed in accordance with the approved guidelines. All animal protocols used in these studies were approved by the Institutional Animal Care and Use Committee at the University of Iowa (#1303056).

Alberts, 2008). The vestibular system of the inner ear in vertebrates senses gravity and other linear and angular acceleration forces. Altered gravity, such as during space flight in astronauts, reduces all movement perceived by the ear to acceleration generated by self-generated or induced movements (e.g., active or passive head shaking). During space exploration, the persistent exposure to microgravity causes reinterpretation of self-generated accelerations during movement (National Research Council, 2011). Astronauts must adapt to this reinterpretation, with appropriate adjustments of their motor functions (Setlow, 2003). Upon return to earth, gravity-related linear acceleration is misinterpreted as induced movement, affecting balance and gaze stabilization for several days (Freeman, 2000; Goldstein, Vernikos, Holmes, & Convertino, 1995; Souvestre, 2004).

Prolonged space flight presents a problem for space exploration as it could lead to altered neuronal connections and reinterpretation of gravity related stimuli as self-generated stimuli (Bruce & Fritzsche, 1997; Legner, 2004; Ronca, Fritzsche, Alberts, & Bruce, 2000). The impact of zero gravity on humans is difficult to study because gravity is constant and uniform on Earth, and the physiological changes induced by the microgravity environment require lengthy exposure to microgravity (West, 2000). To increase gravity, complex and inaccessible hardware must be attained such as large centrifuges that can only add acceleration but do not reduce it (Clement & Pavy-Le Traon, 2004).

Two ways to study these effects are either exposing subjects to long-term microgravity, or constructing model organisms in which gravity is partially or transiently disabled (National Research Council, 2011). Animal models with altered gravity sensation allow researchers to examine effects of various countermeasures on vestibular afferent fibers to form specific connections, establishing normal function. Manipulations of the inner ear, the organ of gravity perception, have been performed by either removing ears or adding extra ears in different orientations in *Xenopus laevis* tadpoles (Elliott, Houston, & Fritzsche, 2015). These manipulations affect the swimming behavior of the animals as well as the distribution of afferents on their target neurons in the central nervous system. Two giant neurons, the Mauthner cells, are a single, large pair of neurons at the level of the ear in the hindbrain of most fish and amphibians (Elliott, Houston, DeCook, & Fritzsche, 2015). The Mauthner cells receive direct ipsilateral, vestibular input from the inner ear and relate this input to a contralateral spinal motor output responsible for the C-start reflex of tadpole, a fast escape reflex with a duration varying between 20–50 ms (Alton, Wilson, & Franklin, 2011; Hale, 2000; Korn & Faber, 2005). Some studies have resorted to using high-speed cameras to image startle responses in tadpoles and other animals (Alton et al., 2011; Hale, 2000; Korn & Faber, 2005; Roberts, Hill, & Hicks, 2000).

To carry out gravity sensing manipulations in tadpoles and monitor them, automated techniques are needed to perform detailed movement pattern analysis. Such techniques include mechanisms to stimulate and record tadpole movement, and control environmental settings during testing. Although previous animal studies simply report escape response swimming behavior as aberrant or normal (Sillar & Robertson, 2009; Soffe, 1991), the experimental apparatus and protocol described here will provide image data that can be used to quantify tadpole movement. To achieve this, we developed an imaging apparatus that uses triggered high-speed imaging to record time-resolved (i.e., at sufficiently high frame rate to

resolve the salient features of tadpole motion), high-resolution image sequences of tadpole movements following stimulation of the C-start response, and temperature control in the test cell to regulate tadpole metabolic function. The experimental apparatus and protocol allows a high degree of experimental control over a large number of tadpoles (potentially tens or hundreds), and multiple tests of each tadpole (biological and technical replication). This paper demonstrates the application and efficacy of the experimental system on tests of control tadpoles with no ear manipulations and tadpoles with asymmetrical input following unilateral ear removal.

Method

Apparatus Setup and Animal Model

The imaging apparatus contains four key elements: (1) a test chamber consisting of a Petri dish containing the tadpoles, suspended in a water bath for temperature control; (2) a water pump and chiller to control the temperature of the water bath; (3) a linear solenoid actuator to provide a controlled stimulus, a forceful tap, to the side of the test chamber for initiating a C-start escape response in the tadpoles; and (4) a high-speed camera to obtain time-resolved, high resolution images of tadpole movements in a test chamber following stimulation of the C-start response with a solenoid trigger.

Subjects—*Xenopus laevis* embryos were obtained through induced ovulation using an injection of human gonadotropin and fertilized with a sperm suspension in 0.3× Marc's Modified Ringer's Solution (MMR). Embryos were kept at 18°C in 90 mm Petri dishes containing 0.1× MMR (diluted from 1× MMR, see below) until they reached stage 46 (Faber, 1994).

Right otic placodes were removed from stage 25–27 embryos in 1× MMR pH 7.6–7.8, diluted from 10× stock (1 M NaCl, 18 mM KCl, 20 mM CaCl₂, 150 mM HEPES) at room temperature under a dissecting microscope (Elliott, Houston, DeCook, et al., 2015; Elliott, Houston, & Fritzsch, 2015). Embryos were kept in 1× MMR for approximately 15 min following ear removal to promote healing prior to their transfer to 0.1× MMR. Healing was confirmed visually as a fusion of ectoderm above the hole left by ear removal. All embryos were kept at 18°C in 90 mm Petri dishes containing 0.1× MMR until they reached stage 46 for a defined healing and growing period.

Test chamber—The Petri dish of diameter 90 mm and height 15 mm was filled with dechlorinated tap water at a depth of approximately 10 mm. A regular hexagonal acrylic frame with side lengths of 30 mm and height 14 mm was placed in the water on the bottom of the Petri dish, providing a boundary to contain a single *Xenopus laevis* tadpole. This allows the field of view of the imaging system to be restricted, and thus increases the spatial resolution of the tadpole images. A similar effect could be achieved by using a smaller Petri dish. Use of a hexagonal frame was found to be more effective than a square frame (which would better fit the field of view) since the animals were more likely to seek shelter in the corners of the square frame.

The Petri dish was placed within a test chamber consisting of an open acrylic rectangular tank of dimensions 180 mm × 180 mm × 60 mm high, as shown in Figure 1, which contains the external water bath for temperature control, while allowing imaging of the tadpoles from below. The tadpoles are temperature sensitive and must be kept in a temperature-controlled environment of 18 ± 0.1°C to prevent extraneous metabolically-driven effects on the C-start response due to temperature fluctuations across experiments. Furthermore, temperature fluctuations above approximately 25° C can create sequences of spontaneous high amplitude C-start escape activity (Sillar & Robertson, 2009). The required temperature control was achieved by placing the Petri dish in a chilled water bath.

The walls and floor were fabricated from 10 mm thick acrylic. To optimize heat transfer between the Petri dish and water bath, and thus ensure adequate temperature control, the Petri dish was elevated above the floor of the test chamber, allowing the cooling water to pass beneath the Petri dish. This was achieved by creating a platform for the Petri dish using a short (35 mm) length of acrylic pipe, with several 10-mm-diameter holes in the pipe wall to allow the circulation of cooling water as shown in Figure 1a.

Control of water flow through the platform—The test chamber contained two inlets and four outlets for the water bath, each of inner diameter 9.5 mm, as depicted in Figure 1b. Two inlets were used in order to minimize turbulence (and thus flow-induced noise) due to the jets of cooling water entering the test chamber. While the noise level and its impact on tadpole response were not quantified, the design objective was to deter transition to turbulence within the inlet tubes or the free jets of water entering the test chamber. The total flow rate of cooling water through all two-inlet tubes was 3 cm³/s. This produces a flow

Reynolds number $Re = \frac{\bar{V}D}{\nu} = 200$ in each tube, based on the mean flow velocity $\bar{V}$, the tube diameter D , and the kinematic viscosity of water $\nu = 1.004 \times 10^{-6}$ m²/s. This is substantially less than that generally understood to cause transition in the internal flow in the tube (White, 2016), and will minimize noise in the free jets of water entering the test chamber (Crow, Crow and Champagne, 1971).

To maintain an equilibrium level of water in the test chamber, flow into and out of the test chamber was regulated with a valve as shown in Figure 2. Careful static adjustment of the valve was sufficient to maintain a consistent level in the tank, and no active level control was needed, thus eliminating the possibility of introducing a confounding stimulus.

Maintenance of constant water temperature using a water chiller and thermal controller—The chilled water was recirculated through a plastic reservoir of approximately 3.5 liters, positioned approximately 0.4 m below the test chamber to allow gravity drainage from the test section. The test section outlets and inlets were connected to the reservoir and pump using clear polyvinyl chloride (PVC) tubing as shown in Figure 2. An Atman PH-500 Skimmer submersible aquarium pump was used to supply water to the inlets through a tubing network consisting of one stage of bifurcations, and back to the reservoir through another tubing network consisting of two stages of bifurcations shown in Figure 2.

Water temperature was regulated using a thermo-electric aquarium chiller (Nova Tec model IPAC-50 W chiller with model TEC-65FADJ temperature controller). The temperature controller employs a thermocouple sensor for feedback control, which was placed in the water in the test chamber and Petri dish. The actual temperature of the water in the test chamber and Petri dish was recorded each minute, using a hand-held digital thermometer (resolution and accuracy of 0.1°C), and frequent checks were done to assess the amplitude of temperature fluctuations in the test chamber. There was no measurable difference between the temperature of the cooling bath and that of the water in the petri dish. The temperature of the cooling bath and the water in the petri dish ranged between 18°C $\pm$ 0.1°C. However, the water source must be kept at temperature below 18°C (specifically 16.2°C for the experiments in this study) to compensate for heat transfer to the chilled water during transport to the test chamber.

High-speed imaging of tadpole movements—The initial contraction and recoil of the tadpole body during the C-start maneuver typically occurs in a period of approximately 20–50 ms (Alton et al., 2011; Hale, 2000; Korn & Faber, 2005). Therefore, high-speed imaging is essential for detailed movement pattern analysis. A high-speed IDT NX-4 camera, which employs a 1024 $\times$ 1024 complementary metal-oxide-semiconductor (CMOS) sensor, was configured with a Nikon Micro-NIKKOR 105 mm f/2.8 macro lens mounted onto the camera using a C-mount adaptor. The camera was oriented vertically and placed underneath the test chamber, approximately 0.6 m from the bottom (yielding a field of view of 100 mm for the experimental setup), in order to view the Petri dish from below, as shown in Figure 3. Images were acquired at a rate of 400 frames per s, with an image exposure time of 1.25 ms and lens aperture of f/5. The *Xenopus laevis* C-start response was found to initiate approximately 25 ms (10 frames) after the trigger, based on the analysis methodology described below. Illumination was provided by an 18 W LED lamp placed directly above the test chamber, as shown in Figure 3, with a softbox diffuser to provide a uniformly lit background. This produced sharp contrast for capturing the two-dimensional shape of the tadpoles as they executed their maneuvers.

Automated generation of tadpole stimuli and camera triggering in unison—To provide a quantitative description of tadpole response to the stimulus, it is necessary to know, precisely, the time of each frame relative to that of the stimulus. For some tadpole modifications, though not for the models used in this study, it is also desirable to capture frames just prior to the stimulus in order to observe the activity of the tadpole at the time the stimulus is applied. The stimulus was provided using a 12VDC linear solenoid activated using a push button switch. Upon activation, the iron core of the solenoid, with a mass of 300 grams, impacts a small (10 cm $\times$ 2 cm) copper-clad circuit board mounted on the side of the test chamber to create a *tap*, which is the stimulus that initiates the tadpole escape response. Contact of the core with the circuit board also closes a circuit that triggers the camera to begin image acquisition. To prevent recoil of the test chamber after impact of the solenoid core, the test chamber was clamped securely to a laboratory bench.

Figure 4 shows the integration of the trigger circuit and the mechanical apparatus. A wire was soldered to the copper-clad circuit board (which contained no other soldered

connections), and another wire was attached to the solenoid core. Both wires were connected to the trigger input of the camera controller such that the circuit was closed and the camera trigger was activated when the core contacted the board. IDT Motion Studio version 2014 software was used to control the camera, which was configured in circular buffer mode. In this configuration, images are acquired continuously and, upon triggering the camera, 100 images are captured after the trigger. The IDT Motion Studio interface also enables images to be retained prior the trigger, but this functionality was not used for this study.

Experimental Procedure and Analysis

All components of the apparatus setup (e.g. camera, light, chiller, etc.) were turned on, and a runtime of approximately 5 min was required for the chiller and pump to achieve equilibrium. For this study, to assess the success of the setup in capturing the C-start response in tadpoles, tadpole stimulus–response tests were performed using 30 different control tadpoles, with a minimum of 1 min between stimuli for a given tadpole. The stimulus was applied with the animal in a voluntary stationary state. In the case when the tadpole did not remain stationary and continued to swim around the test chamber, more than 1 min was needed between trials.

For the characterization test between control and one-ear tadpoles, nine 1-ear tadpoles ($n_m = 9$) with the right ear removed, and six development-stage-matched control tadpoles ($n_c = 6$) were stimulated and imaged as described below. Four stimulus–response trials were conducted per tadpole with at least 1 min between trials to avoid habituation. The response times and directions of the C-start responses (averaged across the four trials) were recorded and compared between the control and one-ear tadpoles.

Tadpole Stimulus–Response Tests—To stimulate and image both control and modified tadpoles, a standard pipette trimmed to provide a 4 mm diameter opening was used to transfer the tadpole to the Petri dish (within the acrylic frame) in the test chamber. After the tadpole became stationary, the pushbutton switch was pressed to activate the solenoid. The camera then recorded the response of the tadpole to the stimulus in a sequence of images obtained at a known time after application of the stimulus. These steps were repeated for the same tadpole for an additional three trials (four stimulus–response trials total, 1 min between intervals to avoid habituation). After the four stimulus–response trials, the tadpole was then removed from the Petri dish and testing of different tadpoles was repeated.

Analysis—Image montages were constructed corresponding to the first 25 frames (e.g. 61.25 ms) after stimulation for each stimulus–response trial, as shown in Figure 5. Because the camera is free-running, the first frame is not exactly synchronized with the stimulus at time $t = 0$, but has an expected time of $t_0 + 1.25$ ms with an uncertainty of ± 1.25 ms (i.e., half the time between image frame acquisitions). All subsequent frames are incremented by intervals of 2.5 ms. Image sequences were manually graded after each trial. Images are graded based on the C-start response time and the direction of each C-start response (recorded as left or right). Response time is defined as the time taken for the head to deflect 10 degrees with respect to the tail, beginning with collinear head and tail axes at the time of the stimulus.

The following protocol was applied, as illustrated in Figure 6, to precisely define the deflection angle:

1. A head axis was defined, as the head does not bend or deflect, by specifying two reliable points associated with the head midline (Fig. 6c). Two head reference points defined the orientation of the head. One point marks the midpoint between the eyes and the second point marks the midpoint of the abdomen, just rostral of the gut, as it attaches to the head. Both points are connected with a thick line segment to show the head axis. The distance between the eyes and the width of the abdomen were not affected by the ear removal (data not shown).
2. A tail axis was also defined in the region of the tail that is not distorted by the traveling wave which starts at the tail base (Fig. 6c). Two tail reference points defined the orientation of the tail: a point halfway between the tail tip and where the tail merges with the abdomen (the tail midpoint), and the tail tip. The tail axis is shown by a thin line segment, extending rostral from the tail, in Fig. 6c.
3. The thin black tail axis line is through all four points in the still animal in Figure 6a. In subsequent frames, the deflection angle, θ , is defined as the angle between the head axis and the tail axis.

By tracking the deflection angle frame by frame, it was possible to estimate the response time of each animal, defined as the time corresponding to the first frame with a deflection angle greater than 10 degrees. The threshold of 10 degrees was empirically determined to be an appropriate proxy to indicate the beginning of the C-start response. At this angle of deflection, the tadpole displays the characteristic and minimally identifiable feature, or kink, with a high level of confidence. Ten degrees serves as the optimal threshold to measure deformation as early as possible, while minimizing uncertainties due to spatial and temporal resolution.

Apparatus Evaluation

Figure 5 illustrates a typical C-start escape response exhibited by a tadpole in response to the solenoid trigger stimulus, captured using the apparatus and protocol described here. Movies illustrating the C-start responses for the control and one-ear tadpoles are available at http://ir.uiowa.edu/mie_pubs/213/. For 117 of the 120 stimulus–response trials performed in controls (Table 1, Animal-Set A), the tadpole exhibited the expected C-start response. Three stimulus–response trials failed to capture a C-start response. We presume that in these trials, the tadpole likely habituated to the stimulus, which is not atypical after multiple, consecutive exposures to the same stimulus even with pauses after each trigger. There was no significant difference in C-start response time ($t(58) = 0.51$, $p = 0.61$) between one-ear tadpoles ($M = 21.98$ ms) and matched controls ($M = 21.68$ ms).

Although there was no observed difference in C-start response time, Figure 7 shows a significant, measurable difference in the behavior of initial direction of the C-start response ($t(58) = 4.00$, $p < .001$): one-ear tadpoles with their right ears removed turned to the right in 97.2% of trials conducted (Table 1, Animal-Set B), while control tadpoles randomly turned to the right or left (Table 1, Animal-Set C). At the animal level (e.g., the aggregation of all

trials for each animal) there was again a significant difference in the behavior of the initial direction of the C-start response ($t(13) = 6.72$; $p < .001$).

Although there was no significant difference in C-start response time between control and manipulated tadpoles, one-ear tadpoles with an asymmetric input nearly always turned in the direction of the removed ear unlike control tadpoles that randomly responded in both directions. Given the contralateral nature of the C-start response mediated by the contralateral projection of the Mauthner cell axon, it is expected that an asymmetrical, right-ear removed ear arrangement would lead to increased firing of the left, fully differentiated Mauthner cell (Elliott, Houston, DeCook, et al., Elliott, Houston, DeCook and Fritzsche, 2015; Elliott, Houston, & Fritzsche, 2015) thus determining the rightward direction of the C-start response.

Discussion

A novel experimental apparatus has been developed to perform the first detailed movement pattern analysis of tadpole startle response. The system, extensively tested with 120 stimulus–response trials, provides a means to perform behavioral assays, which was successfully applied to control and one-ear tadpoles. Specifically, the apparatus imaged and differentiated the C-start response between controls and manipulated, one-ear tadpoles (right ear removed) in response to a consistent stimulus.

While previous animal studies (that focus on the C-start response) simply report swimming behavior as aberrant or normal (Sillar & Robertson, 2009; Soffe, 1991), the experimental apparatus and protocol described in this study can provide image data that can be used to quantify the behavioral response of interest. The final apparatus design presented in this study is the culmination of a series of modifications. Critical steps in applying this methodology rely on proper construction of the temperature-controlled test chamber, configuration of the high-speed camera, set-up of the electromechanical system using the solenoid actuator, preparation of the tadpole embryos, and generation of tadpole stimuli and camera triggering in unison.

The current apparatus and method presented lacks the spatial resolution necessary to examine startle response eye movements and depth-related analysis of swimming behavior. Further research and design will focus on modifying the apparatus to perform a complete, multidimensional analysis of tadpole swimming behavior. Additionally, the current method relies on manual determination of the deflection angle. Future work will be directed to automating this quantification through image analysis and segmentation of the acquired tadpole movement image sequences. Our apparatus can be applied to obtain C-start response data for any tadpole model. Furthermore, there is great promise to extend this experimental apparatus and procedure to other aquatic animal models, such as zebra-fish, to study locomotion as has been done in other animal models (Grillner & Matsushima, 1991).

With animal models, including tadpoles with altered gravity sensation, the experimental approach allows the effects of various counter-measures on vestibular afferent fibers to be studied in detail. The rigorous investigation into the escape response of the tadpole

vestibular system to mechanical and biochemical manipulations, made possible by the experimental apparatus and methods presented in this study, can ultimately contribute to improved understanding of the effects of altered gravity perception on humans and potential countermeasures that can help reestablish normal function following prolonged space travel.

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Fig. 1.

Test Chamber. a) Side view of the test chamber - an acrylic pipe segment is used to support the Petri dish above the bottom of the test chamber floor, with 10 mm holes drilled around the circumference to enhance convective heat transfer on the bottom surface of the Petri dish. b) Top view of the test chamber - the test chamber consists of two inlets on the left and four outlets on the right of the platform.

Fig. 2. Chiller Loop. Chilled tap water is pumped from the reservoir to the test chamber through two inputs. Water from the test chamber returns to the reservoir through a gravity drain consisting of four outlets.

Fig. 3.

2D Drawing of the Camera and Setup. The high-speed camera is mounted below the transparent test chamber. The LED light and diffuser placed above the test chamber and camera provide sharp contrast in the images. Two bars extending from the test chamber are clamped to an adjacent bench to prevent recoil of the apparatus during activation of the solenoid.

Fig. 4. Electromechanical schematic diagram of the integrated system for creating the stimulus and triggering the camera.

Fig. 5.
C-Start Image Sequence. An example sequence of images illustrating the C-start response of a control tadpole with no ear alterations, acquired using a high-speed camera with a frame rate of 400 Hz (2.5 ms between frames). Frame 1 was obtained between 0 and 2.5 ms after the stimulus. Movies illustrating these C-start responses for the control and one-ear tadpoles are available at http://ir.uiowa.edu/mie_pubs/213/.

Fig. 6.

The image grading scheme, and its application using four consecutive images from Figure 5.

a) The stationary tadpole. b) Deflection of at least 10° was used to unambiguously identify the beginning of the C-start response. c) Frame 8 illustrates the methodologies for identifying the head and tail axes used to measure the deflection angle, θ . Reference lines (dashed) are used to define the locations of the head reference points. The head axis is indicated by a thick line, and the tail axis is indicated by a thin line. d) Another subsequent frame in the C-start response sequence reveals continued rotational acceleration of the tadpole.

Fig. 7.

C-Start Response Comparisons. a) Comparison of the proportions of turns between the control (two-ear) and one-ear tadpole trials (Table 1) showed an increased tendency for the one-ear tadpoles to turn in the direction of the removed ear indicating that the normally developed Mauthner cell and its contralateral axon drives the initial response of contracting contralateral muscle fibers thus causing a C-start away from the normal ear. The error bars represent the standard deviations of the proportions of right C-start turns, and the three asterisks indicate a statistical significance of $p < .001$. b) Presentation of individual animal percentages that make up the mean percentages of right turns in 7a.

Table 1**Tadpole models, experiment, animal-set legend**

Animal-set	Phenotype	Tadpoles Used	Trials	Exhibited C-Start Response %	Right-Turn %	Left-Turn %
A	Control	30	120	97.50%	51.30%	48.70%
B	One-Ear	9	36	100%	97.20%	2.80%
C	Control	6	24	100%	45.80%	54.20%

Note. Characteristics of animal-sets used in validation of apparatus. Proportion of right and left turns observed between control and one-ear tadpoles are included.