OPTOMOTOR BEHAVIOUR IN XENOPUS LAEVIS TADPOLES AS A MEASURE OF THE EFFECT OF GRAVITY ON VISUAL AND VESTIBULAR NEURAL INTEGRATION

SCOTT P. PRONYCH, KENNETH A. SOUZA*, ANTON W. NEFF† AND RICHARD J. WASSERSUG‡
Department of Anatomy and Neurobiology, Dalhousie University, Halifax, Nova Scotia, Canada B3H 4H7

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Summary

The ability of aquatic vertebrates to maintain their position requires integration of visual and vestibular sensory information. To understand better how aquatic animals integrate such information, we measured the optomotor behaviour of Xenopus laevis tadpoles raised in growth chambers in microgravity (\(<10^{-3}g\)), normal gravity (1\(g\)), hypergravity (3\(g\)) and on a slowly rotating clinostat (simulated microgravity). The goal of this research was to determine how development in an altered gravitational force field affects the visual- and vestibular-dependent behaviour of tadpoles. This research represents the first time that the optomotor behaviour of an organism raised from fertilization in microgravity has been tested.

Significant differences were observed in the optomotor behaviour among the four gravity treatments. When first exposed to normal gravity, the microgravity-raised tadpoles exhibited the strongest (or most positive) optomotor behaviour, while the 3\(g\) centrifuge tadpoles showed no optomotor response. Some abnormal behaviours (such as erratic swimming, lying motionless and abnormal swimming posture) were observed in the tadpoles raised in altered gravity on the initial day of testing. One day later, the tadpoles raised in hypergravity did not differ significantly in their optomotor behaviour from control tadpoles raised in normal gravity. However, tadpoles raised in microgravity still displayed an exaggerated optomotor response.

One week after the tadpoles had been introduced to normal gravity, there was no longer a significant difference in optomotor behaviour among the different gravity treatments. This convergence of optomotor behaviour by tadpoles from the different treatments reflects the acclimation of their vestibular systems to normal gravity.

Key words: tadpole, Xenopus laevis, neural integration, microgravity, gravity, optomotor behaviour, vestibulo-ocular reflex.

Introduction

Spatial perception in vertebrates involves the sensing of environmental cues using visual, vestibular and proprioceptive systems. The central nervous system then interprets and integrates this sensory information and ‘resolves’ any conflicts in the sensory input. Changes in the environment, such as a decrease in illumination (decreased visual input) or microgravity (altered vestibular and proprioceptive input), can result in failure to integrate spatial information properly. This can degrade an organism’s ability to move and function properly (Daunton and Fox, 1985). In humans and other higher animals, conflicting sensory information about spatial orientation can lead to disorientation and nausea.

One way that visual and vestibular integration is revealed is by the vestibulo-ocular reflexes (Dieringer, 1991; Dieringer et al. 1992). These behaviours are dependent on the vestibular system and serve the important function of stabilizing the visual image on the retina during head or body movement (e.g. if the head moves to the right, the eyes move to the left). The vestibulo-ocular reflex can be suppressed, however, e.g. during tracking of a moving object.

Since the vestibular system can only detect accelerations, the visual system is required for tracking moving objects in cases where there is constant-velocity motion. Positioning of the eyes in response to such visual stimuli is known as an optokinetic nystagmus. The optokinetic response is normally elicited in humans by watching scenery out the window of a moving train or car. As the scenery passes, the eyes oscillate back and forth to track the moving scenery. Tadpoles, such as the ones studied here, exhibit an optomotor response (Wassersug, 1973) which can be induced by surrounding the animal in a cylinder with a visual pattern that rotates around the animal. This response is similar to the optokinetic response.

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*Present address: Space Directorate, NASA Ames Research Center, Moffett Field, CA 94035, USA.
†Present address: Medical Sciences Program, Indiana University, Bloomington, IN 47405, USA.
‡Author for correspondence (e-mail: tadpole@is.dal.ca).
described above, but the animal moves its whole body instead of just the eyes.

Inappropriate visual and vestibular integration is evident in a variety of abnormal behaviours. These have been most extensively studied in aquatic vertebrates and are of particular interest to us in the present study. For example, some organisms will react to a reduction in gravity by exhibiting repetitive righting reflexes (Wassersug et al. 1991a,b; Rahmann and Slenzka, 1994; Wassersug and Izumi-Kurotani, 1993). Amphibians and blinded fish exhibit tumbling, corkscrewing or looping behaviours (von Baumgarten et al. 1972; Rahmann et al. 1990, 1994; Wassersug, 1992; Wassersug et al. 1993; Neubert et al. 1994b; de Jong et al. 1996). In contrast, fish with normal vision do not display this looping behaviour in microgravity (von Baumgarten et al. 1972; Mori et al. 1996; de Jong et al. 1996; S. P. Pronych and R. J. Wassersug, personal observations). Thus, although a fish may sense that the gravity vector has changed, it relies more on visual than on vestibular information for positional cues.

Vestibulo-ocular reflexes of humans have also been investigated in microgravity, on both parabolic and orbital flights (Clement and Berthoz, 1990; Clement et al. 1992). Results from those studies suggest that there is an initial increase in gain in the vestibulo-ocular reflex followed by inhibition after several days in space once the central nervous system adapts. During the period of adaptation, problems in moving or maintaining posture are common (Paloski et al. 1993). Adaptation to altered gravity is thought to involve a reinterpretation of the input from the otoliths to indicate linear translation instead of tilt (Parker et al. 1985; Arrott and Young, 1986; Young et al. 1986, 1992).

The plasticity in the relationship between the vestibular and visual systems can be modified even to the point of reflex reversal (Precht, 1979). Such changes are thought to improve the automatic stabilization of the retinal image during rotation or movement. Importantly, changes in the vestibulo-ocular reflex are thought to be caused by inhibition of vestibular influences (Precht, 1981). In the following study, we manipulated the vestibular input to anuran larvae by exposing them during embryonic development to hypogravity and hypergravity. We used these treatments to examine how altered gravity affects the integration of visual and vestibular information and the resulting perception of spatial orientation by the larvae. In addition, we asked how organisms raised in altered gravity reacted and adapted to a normal gravity environment by looking at changes in their behaviour over time.

To answer these questions, we measured the optomotor response of Xenopus laevis (African clawed frog) tadpoles raised from fertilization in microgravity ($10^{-3} \text{g}$) on the NASA Space Shuttle, simulated microgravity (on a clinostat), normal gravity (1g) and hypergravity (3g) on a centrifuge. Three interrelated areas were examined when the optomotor data were collected: (1) vestibular function; (2) visual function; and (3) the influence of other behaviours such as swimming position or activity on the optomotor response.

This research represents the first time that the optomotor behaviour of an organism raised from fertilization in microgravity has been tested and reveals the plasticity of this response to changes in gravity.

Materials and methods

**Optomotor protocols**

Our optokinetic apparatus consisted of a stimulus cylinder suspended from a small d.c. motor (Fig. 1). The stimulus pattern on the cylinder was composed of six dark stripes (6 mm wide or 23% of total cylinder area, shaded using a 75% black pattern) alternating with six light stripes (20 mm wide or 77% of total cylinder area, shaded using a 2% black pattern) printed on a plastic cylinder of 5 cm diameter. A 41 clear glass, cylindrical dish (20 cm in diameter) was centred under the stimulus cylinder, and a tadpole was positioned at the centre of this dish in a 40 ml glass vial containing 25 ml of biologically conditioned water (water that had previously been used to raise tadpoles) plus 10 ml of Ringer’s buffer. After being given 10 min to acclimate to the vial, each tadpole was tested with the stimulus cylinder rotating for 1 min in either a clockwise or a counterclockwise direction and then tested for another 1 min with the cylinder rotating in the opposite direction. The initial direction of rotation was alternated to compensate for any innate handedness of the tadpoles. On the basis of preliminary trials, the stimulus cylinder was rotated at 10 revs min$^{-1}$ ($60^\circ \text{s}^{-1}$), which evoked a strong and continuous optomotor response from normal Xenopus laevis larvae. At this speed, even the youngest free-swimming tadpoles could

Fig. 1. The apparatus used to elicit an optomotor response in the tadpoles. A motor rotates the spindle at the top. This, in turn, rotates the striped cylinder around the vial which contains the tadpole.
continuously track the stimulus cylinder with no signs of exhaustion. Behavioural observations were recorded with StopWatch Event Recorder computer software (see Appendix). Observations that were made included the tadpoles’ response to the cylinder’s motion (optomotor behaviour), the position of the tadpole in the water column and other ancillary information, such as grossly abnormal behaviour or morphology (see below).

Three behaviours were tracked for determining optomotor behaviour: with, against and still. With, a positive optomotor behaviour, refers to periods when the tadpole swam in the direction of movement of the stimulus cylinder. Against and still are negative optomotor behaviours and refer, respectively, to periods when the tadpole either swam in a direction opposite to the movement of the stimulus cylinder or showed no lateral movement.

The position of the tadpole in the water column was classified into three areas: top, middle and bottom. These each corresponded to approximately one-third of the height of the vial.

Ground-based simulation experiments

This series of trials compared tadpoles raised from fertilization in three different gravity treatments: tadpoles raised in hypergravity on a 3g centrifuge (120revsmin⁻¹), tadpoles raised on a slowly rotating horizontal clinostat (6revs.min⁻¹) to randomize the gravity vector (considered ‘simulated microgravity’; see Neff et al. 1985) and controls raised in approximately normal gravity on a ‘1g’ vertical clinostat (6revs.min⁻¹). The ‘1g’ vertical clinostat controlled for vibrations and slight g-force imparted by the 6revs.min⁻¹ rotation of the horizontal clinostat; we designate this treatment as ‘1g centrifuge’.

Eggs were obtained using standard breeding protocols. Briefly, human chorionic gonadotropin was injected into female *Xenopus laevis* Daudin from adult stock at NASA Ames’s frog colony. The eggs were then artificially fertilized using spermatozoa obtained from the macerated testes of four male *X. laevis*.

The embryos were placed into cultisak (Falcon) chambers (10 bags were cut from a single 0.15 m x 0.23 m chamber unit) and were incubated in 20% Steinberg’s solution (pH 7.4) or 20% frog Ringer’s solution (Neff et al. 1993). Bags containing between 10 and 20 fertilized embryos and approximately 10 ml of solution were heat-sealed with no air space to eliminate air bubbles that could potentially disturb the developing embryos on the clinostats and centrifuge.

Each spawning was tested for percentage fertilization. Typically, spawnings with a fertilization percentage of 95% or higher were used. Bags containing fertilized embryos were placed on the clinostats and centrifuge before first cleavage (typically within 15–20 min after fertilization). Every day, bags were removed individually from the clinostats or the centrifuge for quick observation and removal of unfertilized, dead embryos, grossly abnormal embryos and jelly coats after hatching (typically about 50% of the embryos were removed from the clinostats and the 3g bags by day 3). Approximately 50% of the solution was replaced, and the bags were resealed with no air space. The embryos were incubated at 18°C for the first day and then transferred to room temperature (22–24°C) and maintained on a 14h:10h light:dark cycle from the post-hatching stage (day 3; stage 28–35) until the feeding tadpole stage (day 6; stage 47). The tadpoles were left on the clinostats and centrifuge continuously except for several minutes each day when they were observed. Observations of the involution during gastrulation and measurements of the location of the third cleavage furrow indicated that the embryos responded to the gravitational force field manipulations as expected (Neff et al. 1993).

The centrifuge and clinostats consisted of a styrofoam wheel, 37.2 cm in diameter, with a 1 cm groove in which the bags containing the embryos were placed. Speed of rotation was controlled by a small d.c. motor, and the speed was checked at least once every 12h.

At 6 days after fertilization, the clinostats and centrifuge were stopped. This was designated as day0 and was the first day of optomotor testing. Before testing in the optomotor apparatus, each tadpole was placed in a vial with 35 ml of biologically conditioned water and given 10 min to acclimate. After testing, the tadpole was placed in a container with other tadpoles from its experimental group. One day later (day1), the tadpoles were tested again in the optomotor apparatus. Since the tadpoles were pooled on day0, not all the tadpoles may have been tested again or in the same order. This also means that we did not track the change in behaviour of individuals.

On day0, 147 tadpoles were tested (45 1g tadpoles, 50 3g tadpoles, 52 simulated microgravity tadpoles), and on day1, 115 tadpoles were tested (35 1g tadpoles, 40 3g tadpoles, 40 simulated microgravity tadpoles). Fewer tadpoles were tested on the second day because of manpower constraints and mortality of some of the tadpoles.

Space Shuttle experiments

Four female *Xenopus laevis* were flown on the Space Shuttle and were injected with human chorionic gonadotropin to induce ovulation (see Souza et al. 1995; Black et al. 1996). The resultant eggs were fertilized with a sperm suspension, obtained from the macerated testes of four male *X. laevis* prior to launch. Fertilized eggs (15–30) were placed into each of several growth chambers (a sealed plastic canister) along with 50 ml of Ringer’s solution. The chambers were placed in an incubator with half of the chambers loaded into an onboard 1g centrifuge. The remaining growth chambers permitted embryo development and hatching in microgravity (≤10⁻³g). After the 8-day flight, 13 growth chambers (seven microgravity chambers, six 1g chambers) with live tadpoles were returned to Earth for post-flight behavioural tests.

Post-flight testing was performed 4h (day0), 1 day (day1) and 9 days after landing (days). Up to six tadpoles from each growth chamber were tested in the optomotor apparatus, with two optomotor trials being performed on each individual (one trial for each drum direction). After testing, the tadpoles were...
placed into one of two containers depending on their gravity treatment. The tadpoles were fed intermittently between day1 and dayo of testing to promote normal growth. On dayo, 70 tadpoles were tested (42 microgravity-raised tadpoles, 28 1g centrifuge-raised tadpoles), on day1 71 tadpoles were tested (42 microgravity tadpoles, 29 1g tadpoles), and on day9 65 tadpoles were tested (38 microgravity tadpoles, 27 1g tadpoles).

Ancillary observations

Additional behavioural observations were made and the amount of time that a tadpole exhibited a specific behaviour during the optomotor testing was recorded. The behaviours measured included: looping (a forward, outside loop); corkscrewing (rotation along the longitudinal axis); erratic swimming (swimming with no clear direction or regular pattern); rapid buccal pumping (faster than normal movement of the floor of the mouth); bouncing (moving along the bottom of the vial but unable to swim midwater); motionless (lack of tail movement, either on the bottom or elsewhere, unlike the still observation which only required that the tadpole was not moving with the drum); floating (motionless at the top of the water); upside down (either swimming or lying upside down); swimming head down (when the tadpole is oriented essentially perpendicular to the horizon, with its tail sculling directly above its head, unlike the normal swimming posture where the orientation of the tadpole’s body is diagonal to the horizon); swimming on side (when the tadpole’s dorsal–ventral body axis is perpendicular to the gravity vector); attached to side (most common in young tadpoles which still have their cement glands); slower than drum (swimming in the direction of rotation but at a speed that is slower than the rotational speed of the optomotor cylinder); faster than drum (swimming in the direction of rotation but at a speed that is faster than the rotational speed of the optomotor cylinder); pivots in place (following the optomotor drum while swimming in place); took breath (tadpole went to the surface and successfully took an air bubble in its mouth). Notes were also made of any abnormal external morphology or unexpected swimming behaviours not accounted for in the above list.

To compare the relative behavioural activity of the different experimental groups of tadpoles, a ‘comparative activity number’ was calculated by counting how often the tadpoles switched behaviours – be it optomotor behaviour, position in the water column or one of the other behaviours listed above. Although crude, this number correlates with how active or erratic a tadpole was: that is, a higher number represents a greater frequency of different behaviours.

Statistics

To simplify analysis, tadpoles were grouped by the gravity regime in which they were raised (e.g. microgravity, 1g or 3g). However, owing to logistic constraints, it was not possible to test all tadpoles at the same time. Since the optomotor testing of the tadpoles on each test day took as much as 9h to complete, those tadpoles that were tested later in the day had a longer time to acclimate to any environmental changes (such as Earth’s 1g). This is demonstrated by the fact that, when the optomotor behaviour of the tadpoles on day0 is plotted against the time of testing, there is initially a positive correlation (see Fig. 2; Results). To circumvent the problems of acclimation during each day’s testing of optomotor responses, specimens from different treatments were alternated after every six tadpoles.

No significant differences in developmental stage were apparent after the tadpoles had been staged according to Nieuwkoop and Faber (1956) (analysis of variance, ANOVA, \( P=0.40 \)). Therefore, instead of comparing the tadpoles on the basis of growth chamber (which would have been necessary if the above problems had proved to be significant), we compared the tadpoles on the basis of gravity treatment only.

For the ground-based simulation experiment, six groups were recognized: day0 1g centrifuge; dayo microgravity clinostat; day0 3g centrifuge; day1 1g centrifuge; day1 microgravity clinostat; and day1 3g centrifuge. The experimental grouping is similar for the Space Shuttle experiments, but with a microgravity group instead of a microgravity clinostat group. Only the tadpoles that flew on the Space Shuttle were tested 9 days after initial testing.

The method of optomotor data collection takes the form of dependent underdetermination (i.e. the collection of data that consist of dependent multiple measurements obtained from the same behavioural event), necessitating a multivariate analysis of variance (MANOVA). The statistical software used was SuperANOVA (Abacus Concepts Inc.) on a Macintosh computer. Both univariate and multivariate tests were performed, comparing microgravity, 3g and 1g groups.

Both trials that were recorded for each tadpole on a single testing day were averaged so that only one observation per tadpole was used in the statistical analyses. The averaging of trials was carried out only after a repeated-measures analysis of variance had been performed to determine that there were no significant differences between the trials (clockwise rotation versus counterclockwise rotation and first trial versus second trial).

Regression analysis using SuperANOVA was performed to examine the relationship between the optomotor response of the tadpoles and the time since the Space Shuttle landed (Fig. 2). The main rationale for this analysis was to explore whether the optomotor response of the tadpoles improved over time; thus, only the positive (‘with’) optomotor response was considered. A linear model, the simplest model, was fitted to the data. The ‘best’ regression line was obtained by consecutively adding points and determining what effect the added point had on the overall relationship (18 data points were used for both groups, for a total of four microgravity growth chambers and three 1g growth chambers).

Results

Ground-based simulation experiments

There was no significant difference (MANOVA, \( P=0.774 \))
between the optomotor behaviour of the microgravity clinostat and 1g centrifuge tadpoles on day0 (Fig. 3). However, the 3g centrifuge tadpoles on day0 had a significantly weaker optomotor response when compared with the microgravity clinostat tadpoles (MANOVA, \(P<0.001\)) and 1g centrifuge tadpoles (\(P=0.019\)). In addition, the 3g centrifuge tadpoles showed no significant difference (ANOVA, \(P>0.10\)) between travelling with or against the drum. This means that, when the 3g centrifuge tadpoles did move, their movement was essentially random.

On day1, there was no significant difference (Fig. 3, MANOVA, \(P>0.30\)) in optomotor behaviour between any of the experimental groups. The optomotor behaviour of the 1g and 3g centrifuge tadpoles also improved significantly over that of day0 (Fig. 3, \(P<0.05\) for both groups). There was no significant difference in optomotor behaviour for the clinostat-raised tadpoles (Fig. 3, \(P>0.15\)).

There was no preference for either a ‘left’ (counterclockwise) or ‘right’ (clockwise) drum direction within the groups (repeated-measures ANOVA, \(P=0.36\)). The initial direction of the drum also did not make a difference in the tadpole’s response (\(P=0.50\)).

**Space Shuttle experiments**

There was no significant difference (\(P=0.1618\)) between the optomotor behaviour of the microgravity and 1g tadpoles on post-flight day0 in the multivariate comparison (Fig. 4). However, the microgravity tadpoles did have a stronger response and time of testing. Each point represents the averaged positive response of one individual. Responses are plotted against the time since the landing of the Shuttle. Fitted regression lines, representing the maximal \(r^2\) values for this day of testing, are plotted for both groups (\(N=18\) for both groups). There is no significant difference between the slopes of these two lines (ANCOVA, \(P>0.60\)). The x-axis intercepts are displaced merely because testing of the 1g tadpoles started 22 min after that of the microgravity tadpoles. At later times, to the right of the two regression lines, there is no significant correlation between optomotor response and time of testing.

Fig. 2. Comparison of the positive (with, see Materials and methods) optomotor response versus time of testing for the microgravity (filled circles) and 1g (open circles) tadpoles from the Space Shuttle experiments on day0 of testing. Each point represents the averaged positive response of one individual. Responses are plotted against the time since the landing of the Shuttle. Fitted regression lines, representing the maximal \(r^2\) values for this day of testing, are plotted for both groups (\(N=18\) for both groups). There is no significant difference between the slopes of these two lines (ANCOVA, \(P>0.60\)). The x-axis intercepts are displaced merely because testing of the 1g tadpoles started 22 min after that of the microgravity tadpoles. At later times, to the right of the two regression lines, there is no significant correlation between optomotor response and time of testing.

Fig. 3. Graphical summary of optomotor behaviour from the ground-based simulation experiments on day0 and day1 of testing. Three different experimental treatments were tested: controls raised on a 1g centrifuge, tadpoles raised in hypergravity on a 3g centrifuge, and tadpoles raised on a slowly rotating clinostat. The tadpoles were initially tested on day0 and then retested on day1. The graph shows the average percentage of time that the tadpoles spent travelling with (W) or against (A) the stimulus cylinder, or the time spent still (S). The error bars are \(\pm 2\) S.E.M. (=95% confidence intervals). The dashed lines indicate the only places where neighbouring treatments differ significantly (ANOVA and MANOVA, \(P<0.05\)). Note the general increase in positive optomotor behaviour (increase in W) and the convergence (normalization) of the response for all three treatments after 1 day at 1g.
optomotor response that approaches significance for both the time spent swimming with the drum (ANOVA, $P=0.0625$) and the time spent still ($P=0.0564$). On day 1, the microgravity-raised tadpoles had a significantly stronger optomotor response than the 1g centrifuge-raised tadpoles in the multivariate comparison (Fig. 4, $P=0.0443$). Both groups had a significantly increased optomotor response on day 1 compared with day 0 (Fig. 4, MANOVA, $P<0.05$). On the final day of testing, the microgravity tadpoles’ optomotor behaviour was not significantly different from that of day 1 (Fig. 4, MANOVA, $P=0.2877$). However, the 1g tadpoles’ response increased ($P=0.0612$) to the point that there was no significant difference between the microgravity and 1g tadpoles on day 9 (Fig. 4, MANOVA, $P=0.9152$).

Over the three periods of testing, there was a 15–20% mean increase in positive optomotor behaviour observed for both groups between each day of testing, with the exception of the microgravity tadpoles on day 9. A comparison of the change in optomotor response between both experimental groups over the three periods of testing (Fig. 4) reveals that the 1g tadpoles’ optomotor behaviour continually increased over the 3 days of testing, whereas that of the microgravity tadpoles increased only between day 0 and day 1. However, between day 0 and day 1, values for both groups increased by approximately the same amount for the mean percentage of time spent swimming with the drum (22.4% difference for microgravity tadpoles and 21.2% difference for 1g tadpoles).

In terms of the tadpoles’ position in the water column, there was no significant difference between the microgravity and 1g groups on day 0 and day 1 (Fig. 5, MANOVA, $P=0.1356$ and $P=0.9152$). Note that, initially (day 0 and day 1), the percentage of tadpoles on the bottom decreased throughout the experiment. However, the tadpoles in the 1g and microgravity groups still differed in their position in the water column at day 9.
behaviours, such as swimming upside down, looping, corkscrewing and erratic swimming (see Table 1) were more frequent on day0 – especially in the tadpoles raised on the microgravity clinostat and the 3g centrifuge – than on day1.

The Space Shuttle experiments gave similar results in that most of the abnormal behaviours were observed on day0 of testing (Table 2) and were more common in the microgravity-raised tadpoles. These abnormal behaviours included rapid buccal pumping, erratic swimming patterns, swimming at speeds different from the stimulus cylinder, swimming upside down or remaining motionless. Abnormal swimming for the Space Shuttle tadpoles included some excessive wobbling or shaking when swimming, plus instances of erratic behaviour or being unresponsive to a change in drum rotation. Looping and corkscrewing behaviours were not observed in any of the Space Shuttle tadpoles on any of the testing days.

The comparative activity level of the microgravity tadpoles versus that of the 1g tadpoles approaches significance for day0 (Table 3); however, there was no significant difference between the two gravity treatments for day1 and day9. In terms of differences between comparative activity levels for days of testing, there is no significant difference between day0 and day1. By day9, the comparative activity level of both the microgravity and 1g tadpoles had doubled and was significantly different from that of day1. It is worth noting that the mean comparative activity level on day9 is approximately equal to the maximum comparative activity level observed on day0 and day1 (Table 3).

**Discussion**

*Ground-based simulation experiments*

Tadpoles raised in hypergravity (3g), but tested in normal gravity (1g), showed negative optomotor behaviour on day0 of testing; i.e. they ignored the stimulus cylinder and moved essentially randomly. The clinostat (simulated microgravity) and 1g centrifuge tadpoles that were tested on that day exhibited positive optomotor behaviour. Since all the test groups were raised under similar lighting conditions, there is

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**Table 1. Summary of ancillary observations for 1g, 3g- and clinostat-raised tadpoles for day0 and day1 of testing from the ground-based simulation experiments**

<table>
<thead>
<tr>
<th>Behaviour</th>
<th>1g</th>
<th>3g</th>
<th>Clinostat</th>
<th>1g</th>
<th>3g</th>
<th>Clinostat</th>
</tr>
</thead>
<tbody>
<tr>
<td>Looping</td>
<td>2 (2%)</td>
<td>2 (2%)</td>
<td>1 (1%)</td>
<td>0 (0%)</td>
<td>0 (0%)</td>
<td>1 (1%)</td>
</tr>
<tr>
<td>Corkscrewing</td>
<td>1 (1%)</td>
<td>2 (2%)</td>
<td>3 (3%)</td>
<td>0 (0%)</td>
<td>0 (0%)</td>
<td>0 (0%)</td>
</tr>
<tr>
<td>Erratic swimming</td>
<td>2 (2%)</td>
<td>3 (3%)</td>
<td>4 (4%)</td>
<td>0 (0%)</td>
<td>0 (0%)</td>
<td>0 (0%)</td>
</tr>
<tr>
<td>Bouncing</td>
<td>1 (1%)</td>
<td>3 (3%)</td>
<td>3 (3%)</td>
<td>0 (0%)</td>
<td>0 (0%)</td>
<td>1 (1%)</td>
</tr>
<tr>
<td>Motionless</td>
<td>11 (12%)</td>
<td>26 (26%)</td>
<td>14 (14%)</td>
<td>8 (11%)</td>
<td>8 (10%)</td>
<td>6 (8%)</td>
</tr>
<tr>
<td>Floating</td>
<td>2 (2%)</td>
<td>0 (0%)</td>
<td>0 (0%)</td>
<td>0 (0%)</td>
<td>0 (0%)</td>
<td>2 (3%)</td>
</tr>
<tr>
<td>Upside down</td>
<td>1 (1%)</td>
<td>7 (7%)</td>
<td>5 (5%)</td>
<td>1 (1%)</td>
<td>1 (1%)</td>
<td>1 (1%)</td>
</tr>
<tr>
<td>Attached to side</td>
<td>0 (0%)</td>
<td>0 (0%)</td>
<td>3 (3%)</td>
<td>0 (0%)</td>
<td>0 (0%)</td>
<td>0 (0%)</td>
</tr>
</tbody>
</table>

The table gives the number of trials in which a particular observation was made (percentage occurrence is given in parentheses). All trials were considered, so these numbers do not necessarily reflect the number of specimens that displayed a particular behaviour.
no reason to assume that the visual system of the 3g-raised tadpoles was impaired.

Previous studies have shown that, except for some possible changes in macular synapses, there are no significant changes in the histology of the vestibular organs after exposure to altered gravity (Briegleb et al. 1988; Ross, 1993; Rahmann and Slenzka, 1994; Souza et al. 1994). Therefore, the significant difference in optomotor behaviour between the 3g- and 1g-raised tadpoles is probably related to how the central nervous system integrates visual and vestibular input.

An abrupt reduction in gravity often results in looping by aquatic vertebrates (e.g. Rahmann et al. 1990; Neubert et al. 1994a), and in Xenopus laevis tadpoles this behaviour is manifested as a forward, outside loop; i.e. a somersault (Wassersug, 1992). A few cases of looping were observed in all gravity treatments on day0 of testing (Table 1). Such looping behaviour suggests that the animals ignored visual clues in favour of what the vestibular system ‘reported’. Such discrepancies in visual/vestibular information are evidently handled differently by different animals. For example, in microgravity, blinded fish will loop while fish with visual landmarks do not loop (von Baumgarten et al. 1975; de Jong et al. 1996). However, Xenopus laevis tadpoles will loop after a reduction in gravity (the reduction can be either from 1g to microgravity or from hypergravity to 1g) in the presence of visual cues (Wassersug et al. 1993; Neubert et al. 1994b). Therefore, the Xenopus laevis tadpole’s tendency to discount visual cues when faced with vestibulo-ocular conflict helps to explain the negative optomotor behaviour of the 3g-raised tadpoles on day0.

The low positive optomotor behaviour of the 3g-raised tadpoles (and the 1g-raised tadpoles of the Space Shuttle experiment) may also reflect disturbances in vestibular input due to the Coriolis effect. In a centrifugal field produced by a

Table 2. Summary of ancillary observations for 1g- and microgravity-raised tadpoles for day0, day1 and day9 of testing from the Space Shuttle experiments

<table>
<thead>
<tr>
<th>Behaviour</th>
<th>Day0 Microgravity</th>
<th>Day0 1g</th>
<th>Day1 Microgravity</th>
<th>Day1 1g</th>
<th>Day9 Microgravity</th>
<th>Day9 1g</th>
</tr>
</thead>
<tbody>
<tr>
<td>Looping</td>
<td>0 (0 %)</td>
<td>0 (0 %)</td>
<td>1 (1 %)</td>
<td>0 (0 %)</td>
<td>0 (0 %)</td>
<td>1 (2 %)</td>
</tr>
<tr>
<td>Corkscrewing</td>
<td>0 (0 %)</td>
<td>0 (0 %)</td>
<td>0 (0 %)</td>
<td>0 (0 %)</td>
<td>0 (0 %)</td>
<td>0 (0 %)</td>
</tr>
<tr>
<td>Erratic swimming</td>
<td>3 (4 %)</td>
<td>2 (4 %)</td>
<td>1 (1 %)</td>
<td>0 (0 %)</td>
<td>2 (3 %)</td>
<td>4 (7 %)</td>
</tr>
<tr>
<td>Rapid buccal pumping</td>
<td>13 (16 %)</td>
<td>0 (0 %)</td>
<td>0 (0 %)</td>
<td>0 (0 %)</td>
<td>0 (0 %)</td>
<td>0 (0 %)</td>
</tr>
<tr>
<td>Motionless</td>
<td>28 (33 %)</td>
<td>26 (46 %)</td>
<td>10 (12 %)</td>
<td>14 (24 %)</td>
<td>2 (3 %)</td>
<td>5 (9 %)</td>
</tr>
<tr>
<td>Floating</td>
<td>3 (4 %)</td>
<td>7 (13 %)</td>
<td>3 (4 %)</td>
<td>1 (2 %)</td>
<td>2 (3 %)</td>
<td>0 (0 %)</td>
</tr>
<tr>
<td>Upside down</td>
<td>7 (8 %)</td>
<td>2 (4 %)</td>
<td>2 (2 %)</td>
<td>4 (7 %)</td>
<td>0 (0 %)</td>
<td>1 (2 %)</td>
</tr>
<tr>
<td>Swimming head down</td>
<td>13 (16 %)</td>
<td>2 (4 %)</td>
<td>1 (1 %)</td>
<td>1 (2 %)</td>
<td>2 (3 %)</td>
<td>0 (0 %)</td>
</tr>
<tr>
<td>Swimming on side</td>
<td>4 (5 %)</td>
<td>9 (16 %)</td>
<td>1 (1 %)</td>
<td>2 (3 %)</td>
<td>0 (0 %)</td>
<td>3 (6 %)</td>
</tr>
<tr>
<td>Attached to side</td>
<td>2 (2 %)</td>
<td>0 (0 %)</td>
<td>0 (0 %)</td>
<td>0 (0 %)</td>
<td>0 (0 %)</td>
<td>0 (0 %)</td>
</tr>
<tr>
<td>Slower than drum</td>
<td>7 (8 %)</td>
<td>3 (5 %)</td>
<td>6 (7 %)</td>
<td>1 (2 %)</td>
<td>2 (3 %)</td>
<td>1 (2 %)</td>
</tr>
<tr>
<td>Faster than drum</td>
<td>6 (7 %)</td>
<td>3 (5 %)</td>
<td>6 (7 %)</td>
<td>5 (9 %)</td>
<td>3 (4 %)</td>
<td>2 (4 %)</td>
</tr>
<tr>
<td>Pivots in place</td>
<td>15 (18 %)</td>
<td>5 (9 %)</td>
<td>12 (14 %)</td>
<td>3 (5 %)</td>
<td>0 (0 %)</td>
<td>0 (0 %)</td>
</tr>
<tr>
<td>Took breath</td>
<td>4 (5 %)</td>
<td>4 (7 %)</td>
<td>11 (13 %)</td>
<td>6 (10 %)</td>
<td>9 (12 %)</td>
<td>7 (13 %)</td>
</tr>
<tr>
<td>Abnormal morphology</td>
<td>2 (2 %)</td>
<td>0 (0 %)</td>
<td>1 (1 %)</td>
<td>1 (2 %)</td>
<td>1 (1 %)</td>
<td>1 (2 %)</td>
</tr>
<tr>
<td>Abnormal swimming</td>
<td>1 (1 %)</td>
<td>1 (2 %)</td>
<td>1 (1 %)</td>
<td>4 (7 %)</td>
<td>0 (0 %)</td>
<td>12 (22 %)</td>
</tr>
</tbody>
</table>

The table gives the number of trials in which a particular observation was made (percentage occurrence is given in parentheses). All trials were considered, so these numbers do not necessarily reflect the number of specimens that displayed a particular behaviour.

See text for an explanation of the ‘Abnormal morphology’ and ‘Abnormal swimming’ categories.

Table 3. Summary of the measure of comparative activity for day0, day1 and day9 of testing for the 1g- and microgravity-raised tadpoles from the Space Shuttle experiments

<table>
<thead>
<tr>
<th>Day of testing</th>
<th>Microgravity tadpoles</th>
<th>1g tadpoles</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Mean ± S.E.M.</td>
<td>Range</td>
</tr>
<tr>
<td>Day0</td>
<td>11.6±0.9</td>
<td>3.5–23.5</td>
</tr>
<tr>
<td>Day1</td>
<td>11.6±0.8</td>
<td>2–22.5</td>
</tr>
<tr>
<td>Day9</td>
<td>21.3±1.4</td>
<td>4–43.5</td>
</tr>
</tbody>
</table>

The ‘comparative activity number’ indicates the number of times each tadpole switched behaviours, be it optomotor behaviour, position in the water column or one of the swimming behaviours specified in Table 2.

The ‘Significance’ column reports the P value for a univariate comparison (ANOVA) between the microgravity and 1g tadpole groups.
small-diameter centrifuge at high speeds (120 revs min⁻¹ for the 3g simulation and 60 revs min⁻¹ for the Space Shuttle 1g simulation), there is a gravitational gradient set up across the embryo. Off-axis movement will lead to the left and right vestibular organs experiencing unequal gravitational forces; i.e. a gravity gradient. To minimize the gradient, the tadpole may reduce its swimming behaviour and remain motionless. When the tadpole is removed from the centrifuge, it may still continue to react inappropriately until it adapts to the Earth’s gravitational field, which has essentially no gravitational gradient and minimal Coriolis forces. On the basis of the diameter and speed of rotation in our centrifuges, the largest possible difference in gravitational force between the tadpoles’ left and right vestibular organs was 0.016g. Unfortunately, no information exists on the sensitivity of the vestibular system in tadpoles so it is not known whether such a difference is significant.

Possible evidence of a Coriolis effect is that a high percentage (26%) of the 3g-raised tadpoles reacted to the vestibulo-ocular conflict of the optomotor apparatus by remaining motionless. This motionless behaviour is twice as frequent as that observed in the 1g and simulated microgravity clinostat tadpoles (Table 1) and would account for the negative optomotor behaviour of the 3g-raised tadpoles. Previous research with aquatic vertebrates has shown that, when there is a reduction in gravity, the animal may exhibit looping behaviour, as previously described, or may stop moving and float motionless (Wassersug and Souza, 1990; Neubert et al. 1994a).

On day₁ of testing, there was no significant difference in the positive optomotor behaviour of all three test groups. This indicates that 3g-raised newly hatched tadpoles can achieve a normal optomotor behaviour within 1 day after introduction to normal gravity. This time for acclimation is faster than the 3 days reported previously for the recovery of normal swimming behaviour in Xenopus laevis tadpoles (Rahmann et al. 1992; Slenzka et al. 1994). The difference in recovery times may be due to the length of exposure to hypergravity and the age of the tadpoles. Also, exhibiting normal optomotor behaviour does not necessarily mean that all behaviour will be normal.

Since we only measured optomotor behaviour, it is not possible to know what changes occurred at the neuronal level. Other research, however, suggests that synaptic connections within the macula may decrease with exposure to hypergravity (Rahmann et al. 1992; Ross, 1993; Souza et al. 1994).

The significant increase in optomotor behaviour exhibited by the 3g- and 1g-raised tadpoles between day₀ and day₁ shows that optomotor behaviour varies with stage of development. On day₁, there was also a reduced incidence of abnormal behaviours such as swimming upside down, looping, corkscrewing, remaining motionless, erratic swimming and bouncing (Table 1).

**Space Shuttle experiments**

Positive optomotor behaviour was observed in the microgravity-raised tadpoles on day₀ of post-flight testing.

This optomotor response approached statistical significance (Fig. 4) for time spent still (ANOVA, P=0.0625) and swimming with (P=0.0564) the stimulus cylinder when compared with the behaviour of the 1g-raised (1g centrifuge) tadpoles. By day₁ of post-flight testing, the overall optomotor behaviour of the microgravity-raised tadpoles was significantly stronger than that of the 1g-raised tadpoles (Fig. 4, MANOVA, P=0.0443).

The stronger positive optomotor behaviour of the microgravity-raised tadpoles compared with that of the 1g-raised tadpoles may be related to a difference in how visual and vestibular information was integrated or perceived. In microgravity, the otoliths cannot detect tilt as they do in normal gravity. This leaves two possibilities for how the central nervous system of the microgravity-raised tadpoles treated input from the otoliths: (1) the otoliths’ input was used primarily for detecting linear translation, as suggested in the ‘otolith tilt-translation reinterpretation’ hypothesis (Parker et al. 1985); or (2) the input from the otoliths was primarily ignored (reduced gain).

Regrettably, our testing could not begin until 4 h after the Space Shuttle had landed. This means that any initial reactions to normal gravity may have been missed. However, our ground-based simulation experiment (see above) and previous studies have shown that stable adaptation to 1g from altered gravity requires, at most, a couple of days (Neubert et al. 1994a).

The initial exposure of the microgravity-raised tadpoles to 1g poses several problems for them. These include how to locate the air–water interface in order to inflate their lungs and how to orient properly with regard to the gravity vector. Previous experiments with tadpoles raised in microgravity, but later observed in normal gravity, have revealed vestibular problems such as looping and swimming upside down (Neubert et al. 1994a; Snetkova et al. 1995).

If we assume that the otoliths were used for sensing linear motion (acceleration), then we might expect to observe several behaviours on introduction to normal gravity. First, the stimulus of normal gravity should be interpreted by the tadpole as indicating acceleration or movement in a direction opposite to the gravity vector. A tadpole (with normal spatial orientation) might try to counteract any perceived upward movement by swimming downwards, and looping could result if there were no feedback that the manoeuvre was successful. However, our microgravity-raised tadpoles did not exhibit such behaviour, perhaps because of their confinement in our testing vials.

Second, if the otoliths of the microgravity tadpoles were sensitive to linear acceleration and did not detect tilt, then more erratic swimming and abnormal orientation should have been observed. In particular, if the otoliths of the microgravity-raised tadpoles were sensitive to linear acceleration, then the influence of 1g should result in unexpected vestibular feedback (a vestibular conflict) during movement.

Evidence of a sensitivity to movement in the microgravity tadpoles is apparent in the pivots in place behaviour. While
almost all the 1g tadpoles followed the stimulus cylinder by swimming along the wall of the vial, the microgravity tadpoles moved by pivoting in the centre of the vial for 14–18% of the trials (Table 2) over the first 2 days of testing. By day9 of testing, this behaviour had disappeared, suggesting that the vestibular system of the microgravity tadpoles was initially sensitive to linear accelerations (motion) until the central nervous system reinterpreted the input from the otoliths to signify tilt.

It is important to note that the tadpoles were raised in complete darkness on the Space Shuttle. Since tadpoles raised for a week in the dark require several hours to gain normal vision (S. P. Pronych and R. J. Wassersug, personal observations), this means that all the tadpoles tested early on day0 essentially had limited vision. Thus, spatial orientation of the microgravity tadpoles during the first several hours of testing should have been primarily attributable to the vestibular system. Since the microgravity tadpoles displayed normal orientation and swimming behaviour, this suggests that, even if the otoliths only ‘sensed’ linear acceleration (and not tilt), then this information may have been used in determining the tadpoles’ spatial orientation.

The ability of the microgravity tadpoles to attain and maintain normal spatial orientation is an important observation. Even though these tadpoles had no previous experience with normal gravity, they ‘recognized’ which way was ‘up’ within 4 h after introduction to 1g. This suggests that the microgravity tadpoles may have recognized cues from their vestibular systems and/or from their own buoyancy (e.g. proprioception from the lungs).

The strong positive optomotor behaviour of the microgravity-raised tadpoles earlier on during our testing suggests that these tadpoles relied on their visual system to provide orientational cues and ignored (or lacked) vestibular input. We interpret these results to indicate that the microgravity-raised tadpoles relied more on visual information for determining spatial orientation than their 1g-raised counterparts. This agrees with previous studies that report a gain in optokinetic response in astronauts on return to normal gravity (Clement and Berthoz, 1990).

Why do we see an improvement in optomotor response as testing progressed on day0? One possibility is that the tadpoles were recovering from landing stresses, such as increased gravity and vibration on re-entry. Some acclimation to normal gravity was required of both groups of tadpoles, since the 1g centrifuge was turned off 12 h prior to re-entry. Another possibility is that the tadpoles were acclimating to the light, since all tadpoles were raised in darkness on the Shuttle except for brief periods when they were checked by the astronauts. Since there was no significant difference between gravity treatments for time of acclimation, this suggests that the observed adaptation is not related to differences in visual-vestibular integration.

Both treatment groups also exhibited a significantly stronger optomotor response on day1 compared with day0. This may be attributable to a combination of acclimation to the light, recovery from landing stresses and a more mature tadpole visual/locomotor system.

Some final points concerning the optomotor analysis are that the against optomotor responses never differed significantly between groups and that these responses only accounted for at most 5% of the total testing time. This low incidence of against behaviour suggests that most, if not all, of the tadpoles were able to perceive the rotating drum and follow it correctly. The still response was more common for the first 2 days of testing and, as noted above, this may be attributable to the very young age of the tadpoles. One final point is that, whatever differences in the optomotor behaviour existed on day0 and day1, they had disappeared by day9. Thus, it appears that the behavioural abnormalities in the optomotor response of amphibian larvae resulting from being raised in microgravity are short-lived and are corrected after approximately 1 week in a 1g environment.

The tendency of both the microgravity and 1g tadpoles to spend more time near the bottom or at the top of the water column on day0 suggests that some tadpoles had under-inflated lungs and those tadpoles that had filled their lungs over-inflated them. As the testing progressed, the general trend was that the tadpoles tended to spend more time midwater (Fig. 5). However, by day9, there was a significant difference in the swimming position of both groups, with the microgravity tadpoles spending significantly more time in the middle of the water column (Fig. 5, ANOVA, *P*<0.01). Since a midwater position is normal for *Xenopus laevis* tadpoles, this means that the 1g tadpoles were delayed in taking up a normal swimming posture, that the microgravity tadpoles’ position is abnormal for tadpoles of that particular age or that there are morphological differences (such as in the lungs) that are responsible for the different buoyancies observed (cf. Pronych and Wassersug, 1994; Souza *et al.* 1995).

Looping and corkscrewing behaviours by tadpoles raised in 1g have been commonly observed at microgravity during parabolic flights and when tadpoles raised on a 3g centrifuge were placed in a 1g environment (Wassersug *et al.* 1993; Rahmann and Slenzka, 1994). However, neither of these behaviours was common for any of our tadpoles (Tables 1, 2). The absence of any looping or corkscrewing in the microgravity-raised tadpoles (even while in microgravity on the Space Shuttle) suggests that this behaviour is only elicited when there is a reduction in gravity, not an increase (Rahmann and Slenzka, 1994). The low incidence of looping in our tadpoles also suggests that there were no major morphological, locomotor or vestibular abnormalities in the recovered tadpoles.

The microgravity tadpoles showed a high incidence of rapid buccal pumping on day0. In addition, other behaviours, such as erratic swimming and swimming at speeds different from that of the drum, were observed for these tadpoles. These behaviours are usually indicative of stress. By day1 and day9, these behaviours had decreased in frequency or disappeared altogether.

*X. laevis* tadpoles normally swim continuously by stage 46.
However, high percentages of tadpoles from both gravity treatments were observed to be motionless on day 0 (Tables 1, 2). Note that the motionless observation is not the same as the still optomotor activity. The reduced incidence of motionless behaviour on day 1 and an even further reduction by day 9 suggest that it may simply be a function of the age of the tadpole.

The microgravity tadpoles had a lower incidence of motionless behaviour compared with the 1g tadpoles for all three periods of testing (Table 2). This higher activity level of the microgravity tadpoles was also apparent in the index of their comparative activity on day 0 (Table 3). As mentioned previously, this may be due to stress. Another possibility is that the microgravity tadpoles were more active because of the absence of convection in microgravity. Without convection, oxygen circulation in water is reduced. Movement by the microgravity tadpoles through the water would take them away from oxygen-depleted zones.

The swimming head down behaviour was most common on day 0 in the microgravity tadpoles. A possible explanation is that the microgravity tadpoles were not accustomed to being positively buoyant and had not yet mastered the normal swimming posture. This observation, in conjunction with the observed number of times that the tadpoles took a breath on day 0, suggests that the microgravity tadpoles may have successfully inflated their lungs before testing, possibly even in microgravity. On the basis of previous research (Pronych and Wassersug, 1994; Snetkova et al., 1995), tadpoles prevented from inflating their lungs early in their development require several days to inflate their lungs successfully.

In summary, raising tadpoles under hypergravity and microgravity conditions causes striking differences in their optomotor behaviour compared with that of 1g controls. The observation that the tadpoles recover normal behaviour in 1–9 days suggests that vestibular development and/or visual-vestibular integration were not permanently affected by development in altered gravity. Further experiments are required to determine whether vestibular development is disrupted by longer-term exposure to either microgravity or hypergravity.

Appendix

A three-channel event recorder for behavioural data collection

The StopWatch Event Recorder (SER) software used in this study allows a Macintosh computer to act as a three-channel event recorder. Keys on the computer keyboard register the duration of discrete events. Simultaneous events can also be monitored by logging the length of time that a key is depressed. Up to 20 behavioural observations may be specified and recorded with a time stamp during the course of an experiment. Data that the SER records are: the trial number; ancillary experimental observations fitted to a time line; clock time at the start of the experiment; total time (in s) spent on each activity; percentage of total time spent on each activity; a record of how many times each key was depressed; and a time line that shows when each activity started and stopped during a timing trial.

The SER was originally designed to record behavioural observations of animals in an optokinetic apparatus; thus, it has the facility to verify and record the speed (revs min⁻¹) of a revolving stimulus drum. The SER also has the ability to record information about such features as the stimulus drum design and the direction in which the drum is revolving. These data are recorded along with the results in a standard spreadsheet format.

Accuracy of the SER is to at least 30 μs, with precision to 10 significant digits. Thus, the only source of inaccuracies in the results of the SER is that inherent in the data acquisition itself or, more precisely, in the reaction time of the operator.

Use of the SER is very similar to that of a conventional stopwatch, as the user can start the timer by clicking a key and then click that key or another key to pause the timer. Data on one, two or three activities can be recorded at one time. All timing data are displayed concurrently on the screen as a trial proceeds, so it is possible to monitor the progress of an experiment. Various user-definable macro functions are available to the operator, so that the timing and recording of data can be streamlined and automated. The SER can be optionally used in a countdown mode. In this mode, the SER sounds an alarm and stops the recorder after a user-specified fixed interval. Once a timing trial has been completed, the SER can export data to other programs, for example a statistical or graphing package, so that the results can be further analyzed.

Many people have helped with this research. We particularly thank Tracy Earle, V. Ann King, Luc Bourque and Monika Fejtek for managing the paperwork and providing animal care. Greg Gollan helped design and build various revisions of the optomotor apparatus and its power supplies. Sally Ball looked after the logistics of the experiment by providing the necessary equipment. Tom Stolarik helped with optomotor data collection. This research was supported by the Canadian Space Agency, NASA and the Natural Sciences and Engineering Research Council of Canada.

References


