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

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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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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.* 1991*a*,*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.* 1994*b*; 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 ($10^{-3}\,\boldsymbol{g}$) on the NASA Space Shuttle, simulated microgravity (on a clinostat), normal gravity ($1\,\boldsymbol{g}$) and hypergravity ($3\,\boldsymbol{g}$) 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 4 l 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 $10\,\text{revs}\,\text{min}^{-1}$ ($60\,^{\circ}\,\text{s}^{-1}$), 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 3***g*** centrifuge (120 revs min^{-1}), tadpoles raised on a slowly rotating horizontal clinostat (6 revs min^{-1}) to randomize the gravity vector (considered 'simulated microgravity'; see Neff *et al.* 1985) and controls raised in approximately normal gravity on a '1***g***' vertical clinostat (6 revs min^{-1}). The '1***g***' vertical clinostat controlled for vibrations and slight ***g***-force imparted by the 6 revs min^{-1} rotation of the horizontal clinostat; we designate this treatment as '1***g*** centrifuge'.

Eggs were obtained using standard breeding protocols. Briefly, human chorionic gonadatropin 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 male *X. laevis*.

The embryos were placed into cultisak (Falcon) chambers (10 bags were cut from a single 0.15 m×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 3***g*** 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 14 h:10 h 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 12 h.

At 6 days after fertilization, the clinostats and centrifuge were stopped. This was designated as day_0 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 (day_1), the tadpoles were tested again in the optomotor apparatus. Since the tadpoles were pooled on day_0, 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 day_0, 147 tadpoles were tested (45 1***g*** tadpoles, 50 3***g*** tadpoles, 52 simulated microgravity tadpoles), and on day_1 115 tadpoles were tested (35 1***g*** tadpoles, 40 3***g*** 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 gonadatropin 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 1***g*** centrifuge. The remaining growth chambers permitted embryo development and hatching in microgravity ($<10^{-3}$***g***). After the 8-day flight, 13 growth chambers (seven microgravity chambers, six 1***g*** chambers) with live tadpoles were returned to Earth for post-flight behavioural tests.

Post-flight testing was performed 4 h (day_0), 1 day (day_1) and 9 days after landing (day_9). 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 day_1 and day_9 of testing to promote normal growth. On day_0, 70 tadpoles were tested (42 microgravity-raised tadpoles, 28 1***g*** centrifuge-raised tadpoles), on day_1 71 tadpoles were tested (42 microgravity tadpoles, 29 1***g*** tadpoles), and on day_9 65 tadpoles were tested (38 microgravity tadpoles, 27 1***g*** 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, 1***g*** or 3***g***). 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 9 h 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 1***g***). This is demonstrated by the fact that, when the optomotor behaviour of the tadpoles on day_0 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: day_0 1***g*** centrifuge; day_0 microgravity clinostat; day_0 3***g*** centrifuge; day_1 1***g*** centrifuge; day_1 microgravity clinostat; and day_1 3***g*** 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, 3***g*** and 1***g*** 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 1***g*** growth chambers).

Results

Ground-based simulation experiments

There was no significant difference (MANOVA, P=0.774)

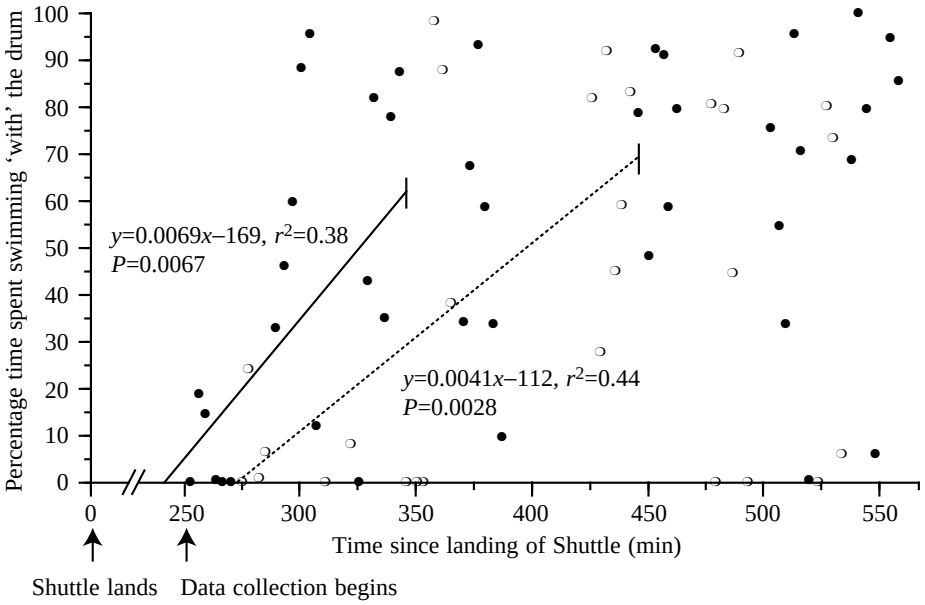

Fig. 2. Comparison of the positive (with, see Materials and methods) optomotor response *versus* time of testing for the microgravity (filled circles) and 1***g*** (open circles) tadpoles from the Space Shuttle experiments on day$_0$ of testing. Each point represents the averaged positive response of 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 1***g*** 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.

between the optomotor behaviour of the microgravity clinostat and 1***g*** centrifuge tadpoles on day$_0$ (Fig. 3). However, the 3***g*** centrifuge tadpoles on day$_0$ had a significantly weaker optomotor response when compared with the microgravity clinostat tadpoles (MANOVA, P<0.001) and 1***g*** centrifuge tadpoles (P=0.019). In addition, the 3***g*** centrifuge tadpoles showed no significant difference (ANOVA, P>0.10) between travelling *with* or *against* the drum. This means that, when the 3***g*** centrifuge tadpoles did move, their movement was essentially random.

On day$_1$, 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 1***g*** and 3***g*** centrifuge tadpoles also improved significantly over that of day$_0$ (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 1***g*** tadpoles on post-flight day$_0$ in the multivariate comparison (Fig. 4). However, the microgravity tadpoles did have a stronger

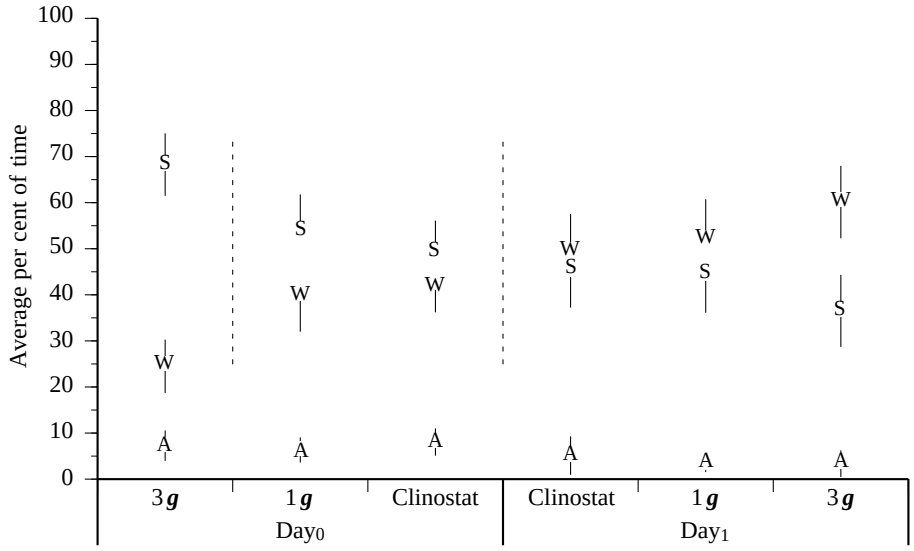

Fig. 3. Graphical summary of optomotor behaviour from the ground-based simulation experiments on day$_0$ and day$_1$ of testing. Three different experimental treatments were tested: controls raised on a 1***g*** centrifuge, tadpoles raised in hypergravity on a 3***g*** centrifuge, and tadpoles raised on a slowly rotating clinostat. The tadpoles were initially tested on day$_0$ and then retested on day$_1$. 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 ±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 1***g***.

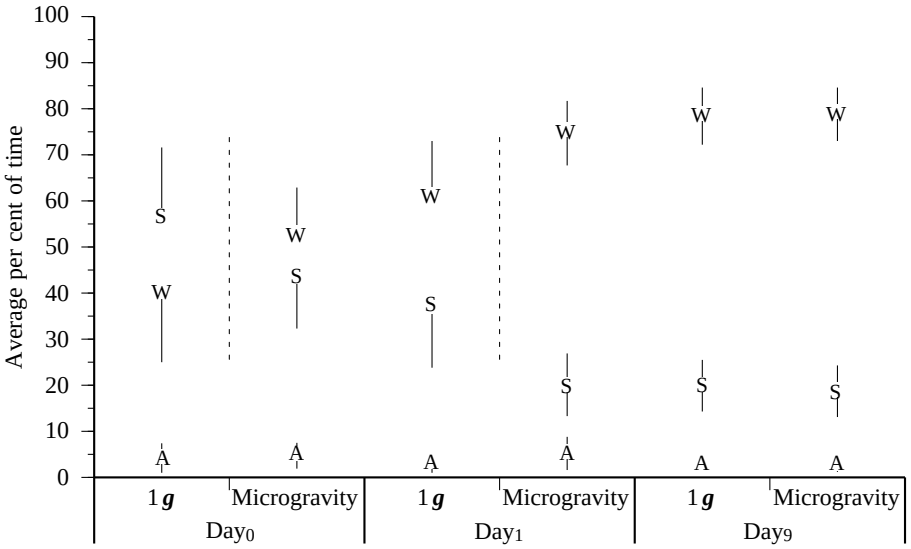

Fig. 4. Graphical summary of optomotor behaviour from the Space Shuttle experiments on day_0, day_1 and day_9 of testing. Two experimental treatments were tested: controls raised on a 1 ***g*** centrifuge and tadpoles raised in microgravity. The tadpoles were initially tested on day_0 and then retested on day_1 and day_9. 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 ±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 that, initially (day_0 and day_1), the tadpoles raised in microgravity have a significantly stronger optomotor response; however, this difference disappears by day_9.

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 1 ***g*** 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 1 ***g*** tadpoles' response increased ($P=0.0612$) to the point that there was no significant difference between the microgravity and 1 ***g*** 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 1 ***g*** 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 1 ***g*** tadpoles).

In terms of the tadpoles' position in the water column, there was no significant difference between the microgravity and 1 ***g*** tadpoles on day_0 and day_1 (Fig. 5, MANOVA, $P=0.1356$ and

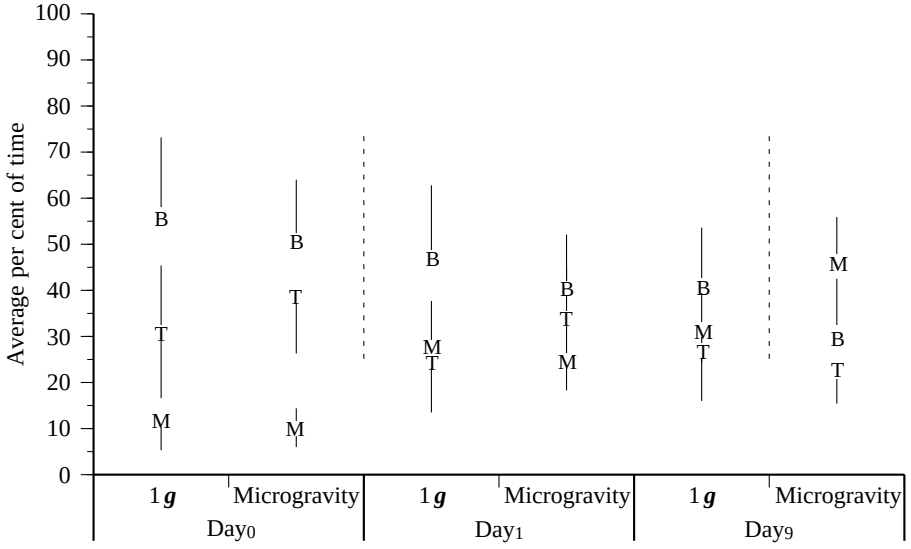

Fig. 5. Graphical summary of the position in the water column for tadpoles from the Space Shuttle experiments on day_0, day_1 and day_9 of testing. Two experimental treatments were tested: controls raised on a 1 ***g*** centrifuge and tadpoles raised in microgravity. The tadpoles were initially tested on day_0 and then retested on day_1 and day_9. The graph shows the average percentage of time that the tadpoles spent swimming on the bottom (B), in the middle (M) or at the top (T) of the water column. The error bars are ±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 that the percentage of tadpoles on the bottom decreased throughout the experiment. However, the tadpoles in the 1 ***g*** and microgravity groups still differed in their position in the water column at day_9.

P=0.6589, respectively). However, both groups did tend to spend more time on the bottom and at the top on day_0 compared with day_1 (Fig. 5). On day_9, the difference in position in the water column of the two groups approaches significance (Fig. 5, MANOVA, P=0.0733). This difference is primarily due to the microgravity individuals spending significantly more time than the 1***g*** controls swimming in the middle of the water column (ANOVA, P<0.01). The swimming position of the 1***g*** tadpoles on day_9 did not differ significantly from that on day_1 (Fig. 5, MANOVA, P=0.9177).

The general trend (see Fig. 2) indicates that the optomotor behaviour of all the tadpoles on day_0 improved as testing progressed, i.e. there was an initial 'period of acclimation'. For the microgravity-raised tadpoles, there was a significant improvement ('acclimation'; see regression line in Fig. 2) over the first 90 min of testing (which ended 343 min after the Shuttle landed; see Materials and methods). The 'period of acclimation' for the 1***g*** centrifuge-raised tadpoles lasted for 190 min after testing started (ending at 444 min after the Shuttle landed) or approximately 90 min longer than that of the microgravity tadpoles. The microgravity tadpoles showed a positive optomotor response as early as 5 h (Fig. 2, at 298 min) after the Shuttle landed; the 1***g*** tadpoles required 6 h (Fig. 2, at 359 min) before showing a positive optomotor response. However, there was no significant difference between the rate of acclimation for the two gravity treatments (ANCOVA, P>0.60).

After the initial 'period of acclimation', there was no significant relationship between response and time of testing on day_0 (microgravity tadpoles r^2=0.032, P=0.4004; 1***g*** tadpoles r^2=0.023, P=0.6750). Similarly, regression analysis for the final 2 days of testing revealed that there was no significant relationship between time of testing and optomotor response (P>0.10).

Ancillary observations

On day_0 of the ground-based simulation, a few specimens showed abnormal behaviour, such as lying upside down or lying motionless, or morphology, i.e. bent tails. Abnormal behaviours, such as swimming upside down, looping, corkscrewing and erratic swimming (see Table 1) were more frequent on day_0 – especially in the tadpoles raised on the microgravity clinostat and the 3***g*** centrifuge – than on day_1.

The Space Shuttle experiments gave similar results in that most of the abnormal behaviours were observed on day_0 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 1***g*** tadpoles approaches significance for day_0 (Table 3); however, there was no significant difference between the two gravity treatments for day_1 and day_9. In terms of differences between comparative activity levels for days of testing, there is no significant difference between day_0 and day_1. By day_9, the comparative activity level of both the microgravity and 1***g*** tadpoles had doubled and was significantly different from that of day_1. It is worth noting that the mean comparative activity level on day_9 is approximately equal to the maximum comparative activity level observed on day_0 and day_1 (Table 3).

Discussion

Ground-based simulation experiments

Tadpoles raised in hypergravity (3***g***), but tested in normal gravity (1***g***), showed negative optomotor behaviour on day_0 of testing; i.e. they ignored the stimulus cylinder and moved essentially randomly. The clinostat (simulated microgravity) and 1***g*** 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

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

	Day$_0$			Day$_1$		
Behaviour	1***g***	3***g***	Clinostat	1***g***	3***g***	Clinostat
Looping	2 (2%)	2 (2%)	1 (1%)	0 (0%)	0 (0%)	1 (1%)
Corkscrewing	1 (1%)	2 (2%)	3 (3%)	0 (0%)	0 (0%)	0 (0%)
Erratic swimming	2 (2%)	3 (3%)	4 (4%)	0 (0%)	0 (0%)	0 (0%)
Bouncing	1 (1%)	3 (3%)	3 (3%)	0 (0%)	0 (0%)	1 (1%)
Motionless	11 (12%)	26 (26%)	14 (14%)	8 (11%)	8 (10%)	6 (8%)
Floating	2 (2%)	0 (0%)	0 (0%)	0 (0%)	0 (0%)	2 (3%)
Upside down	1 (1%)	7 (7%)	5 (5%)	1 (1%)	1 (1%)	1 (1%)
Attached to side	0 (0%)	0 (0%)	3 (3%)	0 (0%)	0 (0%)	0 (0%)

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.

Table 2. *Summary of ancillary observations for 1* ***g****- and microgravity-raised tadpoles for day$_0$, day$_1$ and day$_9$ of testing from the Space Shuttle experiments*

	Day$_0$		Day$_1$		Day$_9$	
Behaviour	Microgravity	1 ***g***	Microgravity	1 ***g***	Microgravity	1 ***g***
Looping	0 (0%)	0 (0%)	1 (1%)	0 (0%)	0 (0%)	1 (2%)
Corkscrewing	0 (0%)	0 (0%)	0 (0%)	0 (0%)	0 (0%)	0 (0%)
Erratic swimming	3 (4%)	2 (4%)	1 (1%)	0 (0%)	1 (1%)	0 (0%)
Rapid buccal pumping	13 (16%)	0 (0%)	0 (0%)	0 (0%)	2 (3%)	4 (7%)
Bouncing	0 (0%)	0 (0%)	2 (2%)	0 (0%)	0 (0%)	0 (0%)
Motionless	28 (33%)	26 (46%)	10 (12%)	14 (24%)	2 (3%)	5 (9%)
Floating	3 (4%)	7 (13%)	3 (4%)	1 (2%)	2 (3%)	0 (0%)
Upside down	7 (8%)	2 (4%)	2 (2%)	4 (7%)	0 (0%)	1 (2%)
Swimming head down	13 (16%)	2 (4%)	1 (1%)	1 (2%)	2 (3%)	0 (0%)
Swimming on side	4 (5%)	9 (16%)	1 (1%)	2 (3%)	0 (0%)	3 (6%)
Attached to side	2 (2%)	0 (0%)	0 (0%)	0 (0%)	0 (0%)	0 (0%)
Slower than drum	7 (8%)	3 (5%)	6 (7%)	1 (2%)	2 (3%)	1 (2%)
Faster than drum	6 (7%)	3 (5%)	6 (7%)	5 (9%)	3 (4%)	2 (4%)
Pivots in place	15 (18%)	5 (9%)	12 (14%)	3 (5%)	0 (0%)	0 (0%)
Took breath	4 (5%)	4 (7%)	11 (13%)	6 (10%)	9 (12%)	7 (13%)
Abnormal morphology	2 (2%)	0 (0%)	1 (1%)	1 (2%)	1 (1%)	1 (2%)
Abnormal swimming	1 (1%)	1 (2%)	1 (1%)	4 (7%)	0 (0%)	12 (22%)

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 day$_0$, day$_1$ and day$_9$ of testing for the 1* ***g****- and microgravity-raised tadpoles from the Space Shuttle experiments*

	Microgravity tadpoles		1 ***g*** tadpoles		
Day of testing	Mean ± S.E.M.	Range	Mean ± S.E.M.	Range	Significance
Day$_0$	11.6±0.9	3.5–23.5	9.1±1.0	3–19	P=0.0643
Day$_1$	11.6±0.8	2–22.5	12.1±1.1	3–26	P=0.7442
Day$_9$	21.3±1.4	4–43.5	21.3±2.3	4.5–50.5	P=0.9979

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 1 ***g*** tadpole groups.

no reason to assume that the visual system of the 3 ***g***-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 3 ***g***- and 1 ***g***-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.* 1994*a*), 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 day$_0$ 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 1 ***g*** to microgravity or from hypergravity to 1 ***g***) in the presence of visual cues (Wassersug *et al.* 1993; Neubert *et al.* 1994*b*). 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 3 ***g***-raised tadpoles on day$_0$.

The low positive optomotor behaviour of the 3 ***g***-raised tadpoles (and the 1 ***g***-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

small-diameter centrifuge at high speeds (120 revs min^{-1} for the 3***g*** simulation and 60 revs min^{-1} for the Space Shuttle 1***g*** 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 0.016***g***. 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 3***g***-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 1***g*** and simulated microgravity clinostat tadpoles (Table 1) and would account for the negative optomotor behaviour of the 3***g***-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.* 1994*a*).

On day_1 of testing, there was no significant difference in the positive optomotor behaviour of all three test groups. This indicates that 3***g***-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 3***g***- and 1***g***-raised tadpoles between day_0 and day_1 shows that optomotor behaviour varies with stage of development. On day_1, 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 day_0 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 1***g***-raised (1***g*** centrifuge) tadpoles. By day_1 of post-flight testing, the overall optomotor behaviour of the microgravity-raised tadpoles was significantly stronger than that of the 1***g***-raised tadpoles (Fig. 4, MANOVA, P=0.0443).

The stronger positive optomotor behaviour of the microgravity-raised tadpoles compared with that of the 1***g***-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 1***g*** from altered gravity requires, at most, a couple of days (Neubert *et al.* 1994*a*).

The initial exposure of the microgravity-raised tadpoles to 1***g*** 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.* 1994*a*; 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 1***g*** 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 1***g*** 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 day_9 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 day_0 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 1***g***. 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 1***g***-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 day_0? 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 1***g*** 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 day_1 compared with day_0. 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 day_0 and day_1, they had disappeared by day_9. 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 1***g*** environment.

The tendency of both the microgravity and 1***g*** tadpoles to spend more time near the bottom or at the top of the water column on day_0 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 day_9, 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 1***g*** 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 1***g*** have been commonly observed at microgravity during parabolic flights and when tadpoles raised on a 3***g*** centrifuge were placed in a 1***g*** 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 day_0. 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 day_1 and day_9, 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 1***g*** 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 1***g*** 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.

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