

The Effects Hypergravity on the Morphology of *Xenopus* Embryos

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ABSTRACT

Early amphibian development is sensitive to both reduced and elevated gravitational force. But later, following gastrulation and neurulation, a critical population of cells must migrate from the dorsal neural tube outward to destinations throughout the body where they differentiate into a wide variety of critical tissues including head cartilage. These cells, the neural crest cells, respond to extracellular cues and signals that guide migration and differentiation in an intricate process that may also be sensitive to altered gravity. We examined the effects of hypergravity on the migration of neural crest cells to form head skeleton cartilage, and on body size in *Xenopus* embryos. To investigate this we centrifuged embryos at 7G or 10G, from yolk plug stage (gastrulation) through five days of development to stage 45 when feeding begins. A control group was placed on the centrifuge. After centrifugation, the embryos were fixed, cleared and stained with Alcian Blue to reveal cartilage. We then captured images for analysis to obtain body and head cartilage measurements. We found that hypergravity retarded the growth of *Xenopus* embryos, possibly via increased load on the cardiovascular system. Surprisingly, it also resulted in significantly larger and more asymmetrical head cartilages, when corrected for body size, but it did not result in a significantly higher frequency of malformations. Our results support the likelihood that hypergravity inhibits body growth and perturbs the formation of neural crest derived head cartilage.

I. INTRODUCTION

Amphibian embryos are small, experimentally convenient, well-known models of vertebrate development. Their early development is known to be altered by exposure to the microgravity (μG). Amphibian embryos exposed to spaceflight μG showed differences in dorsal-ventral body axis, cleavage, gastrulation and neurulation, but these differences are mainly inconsequential [1-5]. For example, Gualandris-Parisot *et al.* (2002) observed changes during cleavage and neurulation, including abnormal segmentation, anomalous closure of the neural tube, decline in cell adhesion, loss of cells, and abnormal movements in the cortical cytoplasm of cells. Yet the larvae that developed had normal morphology and could swim normally [1]. They were later

able to mate and reproduce successfully on earth [6]. Other studies that have involved more limited exposure to spaceflight have shown some changes in the development of the vestibular apparatus in the Japanese newt [7] and the vestibuloocular reflex in the African clawed frog (*Xenopus*) [8]. An exception to most of the published findings is the study published in 1995 by Snetkova *et al.* [9]. These authors found that 11.5 days of spaceflight μG beginning at the tailbud stage induced failure of lung inflation, caudal lordosis, disproportionately long tails, and reduced branchial apparatus in *Xenopus*. Although the vibration and acceleration of the launch and the sudden onset of μG may have been the cause, there are many developmental processes that may be sensitive to changes in gravity occurring over such a long period, for example the migration of neural crest cells.

Simulated μG realized through rotation in a clinostat or rotating wall vessel causes results similar to those seen in spaceflight on early development of *Xenopus*. Changes in cleavage furrow location, blastocoel position, and gastrulation have been shown to be dependably altered, but later regulated, so that normal tadpoles transpire [10-11].

Experimental exposure to higher-than-normal gravity (hypergravity, hG) also produces changes in amphibian embryos during their early development. The observed effects from exposure during embryogenesis are similar to those for μG , affecting embryonic axis formation and the animal-vegetal cleavage ratio (the blastocoel roof becomes thinner). But they are opposite in direction or orientation, and are also regulated back toward normal development [10-13]. Effects on the vestibuloocular reflex are also found (in zebrafish) [14]. Interestingly, if strong hG force (5-30G) is applied for short periods of time very early, during or near the time of fertilization, severe effects are observed in significant numbers of the surviving embryos, including two headedness [15,16]. Others have reported abnormal cement glands and eyes, two-headedness [17], microcephaly, microphthalmia, and diminutive brains and retinas [18]. More recently, Kawakami *et al.* showed that 5G exposure early caused microcephaly, microphthalmia, and reduced cement glands, and that a greater extent of apoptosis than normal occurred in the brain and eyes [19]. These authors also provided evidence that the hG suppressed two genes that are involved in fore- and mid-brain, and eye development (*Xotx2* and *Xag1*, respectively) [19]. It may be that hG has more severe effects early because the cytoplasm has less rigidity and its components more mobility. After cleavage and gastrulation, there are many more cells of smaller volume, and therefore much more microfilament-rich cortical cytoplasm to stiffen cells and support structure. One study examining the effects of hG applied later in *Xenopus* development, however, did report that motor activity for swimming recorded from the spinal nerve ventral roots was affected in burst, duration and rostrocaudal delay by exposure to 3G [20].

Following neurulation, however, there is a critical and sensitive process of epithelial-to-mesenchymal transition for a special population of cells emigrating from the dorsal neural tube, the neural crest cells (NCCs). These cells delaminate from their well-supported residence in the neural tube, become directionally migratory, and respond to intricate signaling processes occurring at their surfaces as they migrate. They also continue to proliferate and become sequentially determined on their way to specific destinations, forming a wide variety of different tissues. In the amphibian head, these cells form, among other structures, the intricate cartilage skeleton that creates the larval feeding and breathing apparatus, including jaws and gill support arches. The migrating NCCs must extend cytoplasmic protrusions, assemble new adhesions, and contract to pull ahead as they travel through the extracellular matrix and cells of the embryonic head. Therefore they may be vulnerable to alterations in the gravity field during the stages of peak migration. There is evidence that cell condensation, gene activation, and secretion of extracellular matrix during chondrogenesis are affected by both μG and hG in cultured mouse embryonic limb [21]. Therefore we predict that moderately high hG applied during the period from the onset of their delamination from the neural tube until the time of feeding at five days (stage 45 according to Nieuwkoop and Faber [22]), will disturb the morphogenesis and perhaps the function of structures largely derived from migrating NCCs, in particular, the head and the branchial skeleton, in *Xenopus*.

II. MATERIALS AND METHODS

a. Embryos And Culture Conditions in the Centrifuge

Adult pairs of *Xenopus laevis* were injected with human chorionic gonadotropin to induce spawning, according to established protocols [23-24]. Embryos were sorted and collected at mid-gastrula stages (yolk plug, stages 10-12 according to Nieuwkoop and Faber [22]). They were dejellied in 2% cysteine in 20% Steinberg's solution (SS), washed five times, and then cultured in 2 ml freshly aerated 10% SS (pH 8.0) in Costar plastic 24-well trays. Each

	Mean Body Length \pm SD (mm)	Mean Snout-Vent Length \pm S D (mm)	Cartilage Defined Head Area (mm ²)
Control	9.7\pm1.0	4.0\pm0.3	3.1\pm0.6
7G	8.9\pm0.6*	3.7\pm0.2*	2.66\pm0.5
Control	9.4\pm0.8	3.9\pm0.4	1.8\pm0.3
10G	8.4\pm0.9*	3.6\pm0.3*	1.3\pm0.3**

Table 1. Body Dimensions of Control and Hypergravity Exposed Tadpoles. Notes: *Significantly different from control ($p < 0.05$); **Significantly different from control, ($p < 1.0 \times 10^{-5}$).

embryo occupied its own well and the tray was covered with an unsealed lid (allowing air exchange). Two trays containing 24 embryos each were placed on the carriers of a swinging rotor, which was spun for five days in an Eppendorf 5810 R centrifuge. The speed was set at 200 RPM for 7G at mid-tray, or 240 RPM for 10G at mid-tray. The centrifuge equilibrated quickly and maintained $22^{\circ}\pm 1^{\circ}$ C. One or two control trays with 24 embryos each were placed on top of the closed centrifuge in order to equalize exposure to vibrations as nearly as possible. The control tray was exposed to the temperature of the room, which was $22^{\circ}\pm 1.5^{\circ}$ C. During the experiment the centrifuge was stopped briefly on the second and third days and the SS was changed to fresh, aerated SS, and any dead embryos were noted and removed. Oxygen levels remained at 5 mg/L or above and no mold or bacterial contaminations were found during the experiments.

b. Fixation, Staining, and Analysis

At the end of each five-day experiment, the centrifuge was stopped. The SS was pipetted out and two ml of 4% paraformaldehyde were added to fix the embryos. The embryos were then photographed using a Leica MZ16 stereomicroscope with digital camera and computer. Measurements of total body and snout-to-vent lengths were made and recorded. Then the embryos were cleared and stained for cartilage with Alcian Blue according standard procedures [25-26]. They were photographed from the ventral

side and the images were analyzed with Openlab (Improvision, Inc.), Optimas (Media Cybernetics, Inc.), or Image-Pro (Media Cybernetics, Inc.) software. Measurements of area were made by tracing around perimeters, including each individual cartilage and the entire collective cartilage defined head area (CDHA). We corrected each measurement for body size difference by calculating each length or area as a percent of the CDHA. The centrifuged and control group measurements were compared using T-tests.

III. RESULTS

a. Body Dimensions

Following incubation of *Xenopus* embryos at 7G, 10G or 1G (control) from gastrulation until feeding tadpole stage (five days), we measured body dimensions. Table 1 displays the results. Exposure to 7G and 10 both reduced the size of *Xenopus* larvae significantly. The 7G condition resulted in larvae that were 8% shorter in total body length and 7.5% shorter in snout-vent length. The 10G condition reduced these lengths by 10.6% and 7.7%, respectively. Thus the effect was more apparent on the tail, which grows more in the longitudinal dimension, than on the head/trunk region. Another measure of size, the cartilage-defined head area (CDHA), also showed reduction in hG. Although the 14% reduction seen at 7G was not significant, the 28% reduction in area in the 10G condition was highly significant.

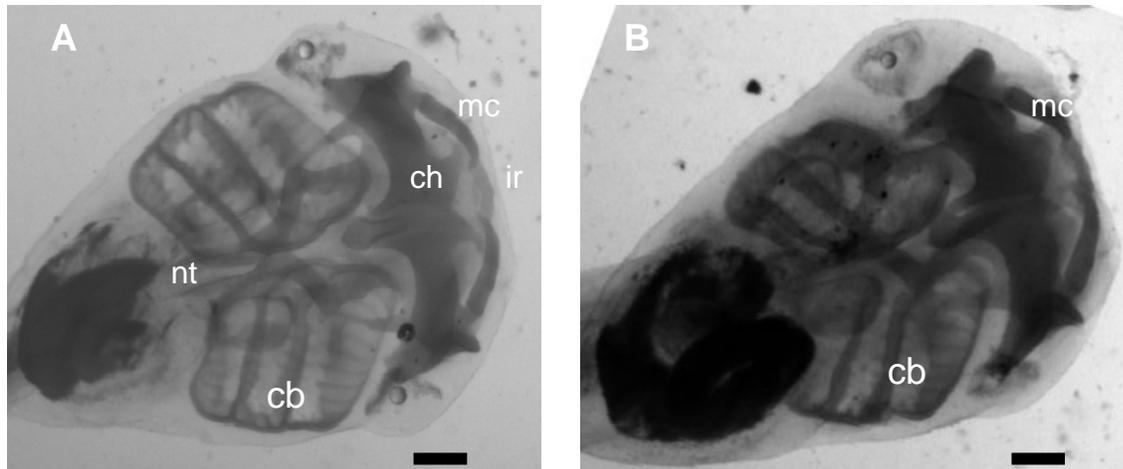


Figure 1. Alcian Blue-stained larvae heads after five days of development. A. Control. The notochord (nt) and head cartilages are well-formed and symmetrical. B. Skull exposed to 7G from gastrulation through five days. Malformations were rare but in this specimen the ceratohyal and the ceratobranchials are narrower and smaller on the left side, with reduced or missing elements. Bars = 0.5 mm. cb=ceratobranchial, ch=ceratohyal, ir=infrarostral, mc=Meckel's cartilage, nt=notochord.

b. Individual Head Cartilage Dimensions and Asymmetry

We also analyzed the lengths of each Meckel's cartilage and the infrarostral, the diameter of the notochord, and the areas of each ceratohyal and each ceratobranchial basket (Figure 1). During development at 7G, the left Meckel's cartilage was significantly longer, and the right ceratohyal and left ceratobranchial were significantly greater in area than in the 1G controls. At 10G (Figure 2) all the cartilages were significantly larger except for the right ceratobranchial basket.

The symmetry in length or area between corresponding right and left cartilages was evaluated by calculating the absolute value of the difference between right and left, and then comparing these differences for hypergravity and control groups. The measurements are displayed in Table 2. We found that at 7G, the Meckel's cartilage lengths and the ceratobranchial areas were more than three times more asymmetrical than in controls. The ceratohyal cartilage areas were also more asymmetrical in area, but the difference was not significant. However at 10G, none of the means of right-left difference showed significant differences.

c. Mortality, Malformations and Activity Level

Dead embryos were removed from the experiment at the 24 and 48 hour water change stops. Despite the physical load from hG, few embryos died. In the 7G experiments, 7/92 control (8%) and 5/88 hG (6%) embryos were removed. In the 10 G experiment, none of the control and 5/48 (10%) of the hG embryos were removed.

Malformations that we observed included skeletal, eye, intestine and tail defects, but no two-headedness. The most common defects were in the number, alignment and angle of the ceratobranchials. We categorized the malformations as mild or severe and the results are shown in Table 3. It was clear from the results that the incidence was not unusually high for *Xenopus* and that neither 7G nor 10G increased it.

Embryos that developed in hG were noticeably less active than controls upon observation at the end of the 5-day experiment. Although they could swim, their swimming was much less frequent and their swimming episodes were short. The effect was more pervasive at 10G than 7G.

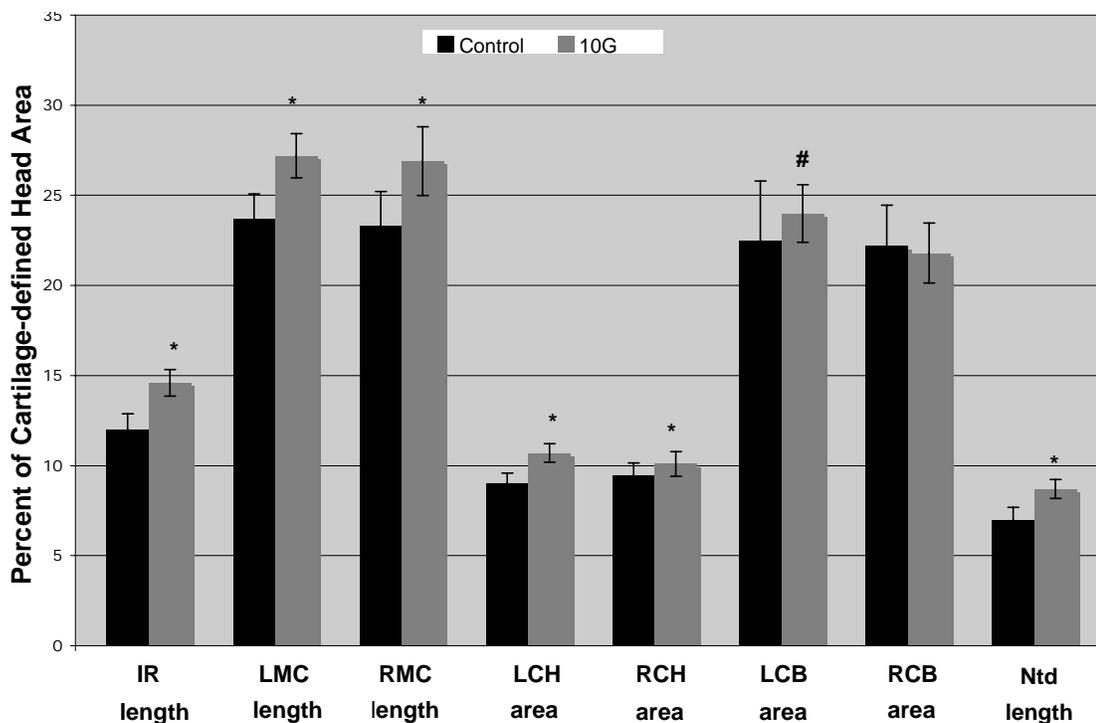


Figure 2. The length of the infrarostral (IR), left and right Meckel's (LMC and RMC, the width of the notochord (Ntd), or the area of the left and right ceratohyal (LCH and RCH) and left and right ceratobranchial baskets (LCB and RCB) are plotted here as the means of 25-30 percents with error bars indicating standard deviations. Except for the right ceratobranchial, all cartilages were significantly larger proportions of cartilage defined head areas. Notes: *Significantly larger than control ($p < 0.001$); #Significantly larger than control ($p < 0.01$).

IV. DISCUSSION

We exposed *Xenopus* embryos to 7G or 10G from gastrulation (approximately 9-12 hours of development) until feeding tadpole (five days) when the head skeleton must begin to function in jaw movements as well as respiration. Our experiment thus excludes any effects of hG on the egg, zygote, cleavage, or the early events of gastrulation. Within a few hours of the beginning of centrifugation the germ layers were largely established and NCC migration had begun. We observed a significant inhibition by hG of growth in body length, which was more pronounced at 10G than at 7G. We also observed that the CDHA, a measure of the general size of the head and branchial region, was reduced in hG, and more so at 10G than at 7G. Since the embryos in each experiment were all from the same spawning and the temperature was controlled, it seems probable that hG

affected the physiology of growth. The formation and growth of the body as a whole during the period from gastrulation to 5-day tadpole would depend largely on the absorption and conversion of yolk material. This process in turn, would depend heavily on the heart following its formation and onset of function, and its ability to circulate blood. The heart begins to beat at stage 33-34, about 45 hours of development. Thus the centrifuged embryos required about 85 hours of cardiac output in 7G or 10G instead of 1G for controls. Regardless of position, the increased gravitational force would load the heart with extra resistance to overcome, potentially limiting dispersion of yolk nutrients via the circulation. If the hG-exposed developing heart does work against higher resistance, it may be able to respond with some degree of hypertrophy or higher pace. A morphological study of the response of adult rat heart muscle to 2G for fourteen days' exposure revealed greater

	Meckel's Cartilage Mean of Differences in Length \pm Variance (nm)	Ceratohyal Area Mean of Differences in Area \pm Variance (nm ²)	Ceratobranchial Mean of Differences in Area \pm Variance (nm ²)
Control	14\pm0.25	10\pm10	10\pm0.5
7G	45\pm0.5*	15\pm0.5	36\pm0.8*
Control	49\pm2	30\pm0.6	69\pm5
10G	39\pm4	26\pm0.4	48\pm1

Table 2. Asymmetry: Differences between right and left head cartilages. Note: *Significantly larger than control, $P < 0.0002$.

	Total Number of Embryos	Number of Mild Abnormalities	Number of Severe Malformations
Control	37	2 (5%)	5 (14%)
7G	57	2 (4%)	2 (4%)
Control	42	0	12 (29%)
10G	37	0	8 (22%)

Table 3. Incidence of Malformations.

myofiber areas and mitochondria with signs of fatigue typical of hypertrophy [27]. Evidence of these responses in *Xenopus* tadpoles would be available by carefully examining heart rate, size, and fine structure, a goal for future research. We did observe a lower level of swimming activity among centrifuged tadpoles at the end of the experiment which would be predicted if circulation is a limiting factor. This change in swimming activity level may also be related to the effects of hG on ventral root neural activity seen by Böser and Horn [20].

We found that individual cartilages in hG-exposed tadpoles, although smaller, were actually a larger proportion of body size than controls. At 7G, all cartilage mean lengths or areas were slightly larger, though only three were significantly so. But at 10G, seven of the eight cartilages were significantly larger. This result seems paradoxical considering the general inhibition of growth. It suggests that skeletal

chondrogenesis was not inhibited in proportion to other tissue development, yielding smaller larvae with relatively large skeletons. The head skeleton is formed mainly by migratory cranial NCCs whose differentiation as cartilage depends on paracrine factors secreted by the tissue environment through which they migrate. And if this environment has reduced dimensions because of hG-induced general growth inhibition, it might be surmised that the growth factor gradients influencing cranial NCCs might be steeper and able to induce stronger proliferation and differentiation locally. Theoretically, this could increase their relative size. The fact that the notochord, a tissue not formed from NCCs, was also significantly larger, indicates an effect on all elements of the skeleton, not only neural crest.

The number of malformed embryos that we observed at the end of the experiments varied considerably with the

spawning, however there was no effect of hG on the percent with malformations. Interestingly, there were more severe malformations and fewer mild malformations at 10G than at 7G, though the number of malformations was so small that this difference may be due to chance. We also found that hG increased the degree of asymmetry of head cartilages at 7G, though not at 10G. This result might be explained if mortality were higher in 10G experiment, the asymmetric embryos having died, leaving the more symmetrical ones alive to survive to the end of the experiment. Mortality was fairly low in the 7G experiments (8% for control and 6% for hG) but was slightly higher in the 10G experiment (0% control, 10% hG). If the 5 dead embryos that were removed (2 at 24 hours and 3 at 48 hours) from the total of 48 embryos had a large amount of asymmetry in their cartilages, the measured asymmetry would have been higher.

We conclude that hG inhibits growth of *Xenopus* embryos from gastrulation to feeding tadpole stage, possibly via increased load on the cardiovascular system. Surprisingly, it also results in significantly larger and more asymmetrical head cartilages, when corrected for body size, but not in a significantly higher frequency of malformations. The results are consistent with our prediction that NCC migration is sensitive to hG, but further experiments will be required to confirm this. Careful assessment of the relative concentrations and distributions of growth factors known to be important for cranial NCCs will be needed to understand the consequences of hG in amphibian skeletal development.

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