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# Hypoxic conditions alter developing branchial arch-derived structures in zebrafish

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## Abstract

**Background**: Previous epidemiological findings have implicated hypoxia as a risk factor for craniofacial defects including cleft lip, microtia and branchial arch anomalies. This study tests the hypothesis that hypoxic exposure results in craniofacial shape variation in a zebrafish model. **Methods**: Three sets of zebrafish embryos were raised in uniform conditions with the exception of dissolved oxygen level. At 24 hours past fertilization (hpf) embryos were placed in hypoxic conditions (70% or 50% dissolved oxygen tank water) and compared to unexposed control embryos. After 24 hours of exposure to hypoxia, the embryos were incubated under normoxia. Larvae were collected at 5 days post fertilization (dpf) and stained for cartilage. Images were taken of each specimen and subsequently landmarked to capture viscerocranial morphology. A geometric morphometric analysis was performed to compare shape variation across groups. **Results**: The mean branchial arch shape of each exposure group was significantly different from controls ($p<0.001$). Principal components analysis revealed a clear separation of the three groups, with controls at one end of the shape spectrum, the 50% hypoxia group at the other end, and the 70% hypoxia group spanning the variation in between. **Conclusions**: This experiment shows that hypoxia exposure at 24hpf is capable of affecting craniofacial shape in a dose-dependent manner. These results may have implications not only for high altitude fetal health, but other environments, behaviors and genes that affect fetal oxygen delivery.

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## INTRODUCTION

There are multiple lines of evidence indicating that hypoxia can alter craniofacial development. Most research has focused on the midface and the effects of reduced oxygen on the development of orofacial clefts. For example, maternal cigarette smoking has been shown to increase the risk of oral clefts in offspring [1], the incidence of clefting appears to be elevated in certain high-altitude populations [2], and cleft mouse models show increased incidence of clefting after hypoxia exposure [3]. There is also evidence that hypoxia can affect a broader range of structures comprising the craniofacial complex, including branchial-arch related morphology [2,4]. For example, the observed prevalence rate for microtia is five times greater for individuals living at high-altitude compared with those living at low altitude in the same general region, suggesting that development of the first and second branchial arches is susceptible to hypoxia [5].

It is critical to understand the multitude of the factors capable of altering the underlying developmental architecture ultimately resulting in craniofacial malformations. Animal models are tools that help determine which factors warrant further scrutiny. Zebrafish (*Daniorerio*) provide a relatively quick and inexpensive way to screen for factors involved in phenotypes of interest [6]. While zebrafish are most often used to explore genetic questions, they also provide an opportunity to test environmental effects such as oxygen availability on phenotypic outcomes. Previous experiments with zebrafish in low-oxygenated water have resulted in general growth retardation and developmental delay [7,8].

The present experiment focuses on the zebrafish viscerocranium. The structures comprising the viscerocranium are derived from branchial arches. The branchial arches consist principally of neural crest cell-derived mesenchyme and are separated by endodermal pouches. In fish, the first and second branchial arches give rise to various structures of the craniofacial complex; the first arch gives rise to Meckel's cartilage and the dorsal palatoquadrate, while the second arch forms the ceratohyal, basihyal, interhyal and hyosimplectic [9]. In mammals, first and second arch derivatives include parts of the middle ear, mandibular process, maxillary process and part of the hyoid bone [10].

In this study, two hypotheses about hypoxia and craniofacial shape variation are tested with zebrafish. The first hypothesis contends that hypoxia will alter the shape of first and second branchial arch-derived structures in developing zebrafish embryos. The second hypothesis states that hypoxic stress will result in different levels of phenotypic perturbation in a dose-dependent manner.

## MATERIALS AND METHODS

### Zebrafish Maintenance

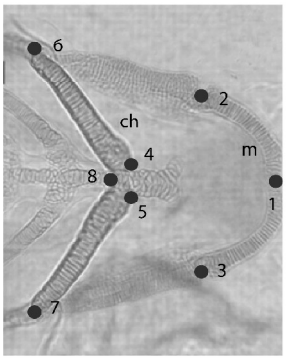
Adult wildtype zebrafish AB* were maintained in a zebrafish housing system (Aquaneering Inc.). Adult fish are maintained at 25-27°C with reconstituted Reverse Osmosis Water (pH 7.0-7.5; Conductivity: 650-750 µS/cm; Dissolved Oxygen: 7.5mm Hg) in 14 h: 10 h light: dark photoperiod. Male and Female adult zebrafish were set up the day before and embryos were collected in the morning and staged according to Nuesslein-Volhard et al [6]. Embryos were transferred to petri dishes containing E3 solution (5mM NaCl, 0.17 mMKCl, 0.33 mM CaCl2, 0.33 mM MgSO4) and incubated at 28.5°C.

### Specimen Preparation and Data Collection

Embryos from a single clutch were divided into three different conditions: negative control/normal $O_2$ level(6.5-6.6mm Hg, which was considered 100% $O_2$), 50% $O_2$ level, and 70% $O_2$ level)at 24 hours post fertilization (hpf) for a period of 24 hours. Oxygen levels were manipulated by bubbling nitrogen gas and measuring dissolved oxygen level with a dissolved oxygen meter (SonTek/YSI modell 58, Fisher Scientific) in E3 media. Embryos were added to the deoxygenated E3 and placed into 50ml conical tubes and sealed, thereby reducing the amount of oxygen transfer in the process. The embryos were incubated at 28.5°C and after the 24 hours the larvae under hypoxic conditions were returned to normoxic E3 media.

At 120hpf, a portion of specimens from each group were sacrificed with tricaine treatment (MS-222, Sigma) and fixed in 4% paraformaldehyde. The five-day time point(120 hpf) was chosen because it is within a developmental window where the visceral cartilages are well developed but just prior to the stage when feeding occurs. The mechanics of feeding could introduce mechanical influences on shape that would confound our analysis. The skin surrounding the anterior half of the embryo and the brain were removed from each specimen. At this time the eyes were also removed as they interfered with the visualization of certain craniofacial structures. After dissection the specimens were dehydrated with a series of ethanol treatments and stained for cartilage with Alcian blue (0.1% Alcian blue dissolved in 80% ethanol/20% glacial acetic acid) followed by the addition of sodium borate to neutralize the acid [11].

Ventral images of the branchial arches were captured using a camera-mounted microscope, similar to the methods used by Lopez-Romero and co-workers [12]. Subsets of specimens from each of the three experimental rounds were included in the data collection for a total sample size of 35 (70% $O_2$,n=19; 50% $O_2$,n=9; normal controls, n=7). Each larva was held in place with pins through their caudal end to minimize orientation error in the images. Eight two-dimensional landmarks were collected on first and second gill arch-derived structures by a single digitizer using tpsDIG2 [13] and the corresponding *x,y* coordinates saved for analysis. Landmark locations were based on those presented in Alberston and Yelick [14] which are listed and presented in Figure 1.Institutional ethical approval was obtained prior to any animal experimentation (University of Pittsburgh IACUC Protocol #0409348)




Landmark descriptions:

1: Center of first arch

2: Left junction of Meckel's cartilage and the palatoquadrate

3: Right junction of Meckel's cartilage and the palatoquadrate

4: Left anterio-lateral junction of ceratohyal with the basihyal

5: Right anterio-lateral junction of ceratohyal with the basihyal

6: Left lateral most point of the ceratohyal

7: Right lateral most point of the ceratohyal

8: Midline point of the posterior ceratoyhyal junction with the basihyal

**Figure 1**: Landmark key.

### Statistical Shape Analysis

A geometric morphometric (GM) approach was used to analyze the landmark coordinate data. When performing a GM analysis, the first step after landmark collection is a Procrustes superimposition of the entire coordinate dataset to align all the landmarks in the same coordinate system, which makes all specimens comparable to one another [15,16]. The Procrustes coordinates were then regressed on centroid size (pooled within-groups approach) to remove any variation in shape that is due to size (i.e. allometry). The residuals from this regression were then used as the shape variables for the ensuing multivariate statistical tests. All analyses were performed in MorphoJ 1.04a [17].

To test the first hypothesis, the branchial arch mean shape from each reduced $O_2$ group was compared to the control group. This was accomplished by computing the Procrustes distance from each group mean to the control mean; statistical significance was determined via a 5000-round permutation test. To test the second hypothesis of a directional relationship between hypoxia levels and shape variation, both a principal components analysis (PCA) and a canonical variate analysis (CVA) were applied to the shape data. The PCA reduces the multivariate data to a set of tractable axes representing the major modes of shape variation; the first few principal components (PCs) represent the axes containing the greatest amount variation in the sample, with each subsequent PC representing a lesser amount. By using GM methods, the shape variation these axes display can be modeled with wireframes that are warped using a thin plate spline technique [18]. CVA is a similar data-reduction method, but where PCA reduces the data without any assumptions regarding group membership, in a CVA the groups are specified *a priori*. In effect, the CVA seeks to maximize the shape variation that most effectively discriminates among groups [16].

**Table 1:** Procrustes distances between the mean shapes of each group and their associated p-values as determined by a permutation test (5000x).

| Comparison Groups | Procrustes Distance | p-value |
|---|---|---|
| Control vs. 70% Oxygen | 0.162 | <0.001 |
| Control vs. 50% Oxygen | 0.243 | <0.001 |
| 70% Oxygen vs. 50% Oxygen | 0.090 | 0.0072 |

## RESULTS

Results of the mean shape comparison between the hypoxia groups and the control group are listed in Table 1. Based on the permutation test, the mean shapes of both the 70% oxygen group and the 50% oxygen group are statistically significantly different compared to that of the control group ($p<0.001$). The two experimental groups were also statistically dif-

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ferent from each other in terms of shape ($p = 0.0072$). The centroid sizes of the experimental groups were both statistically lower compared to the control group (70% versus control, $p = 0.013$; 50% versus control, $p = 0.002$).

The results of the PCA and CVA are depicted in Figure 2. The scatter plot of the first two PCs indicates that the greatest axis of variation, PC1, corresponds to hypoxia level. PC1 accounts for 57.4% of the shape variation in the dataset. The control shapes cluster at one end of the plot; the 70% oxygen specimens are largely in the middle with the 50% at the opposite end from the controls. The 70% oxygen group shows the widest range of shape variation along PC1, spanning nearly the entire axis. The variance in scores associated with PC1 did not differ statistically among the three groups ($p > 0.05$; non-parametric Levene's test for equality of variances).

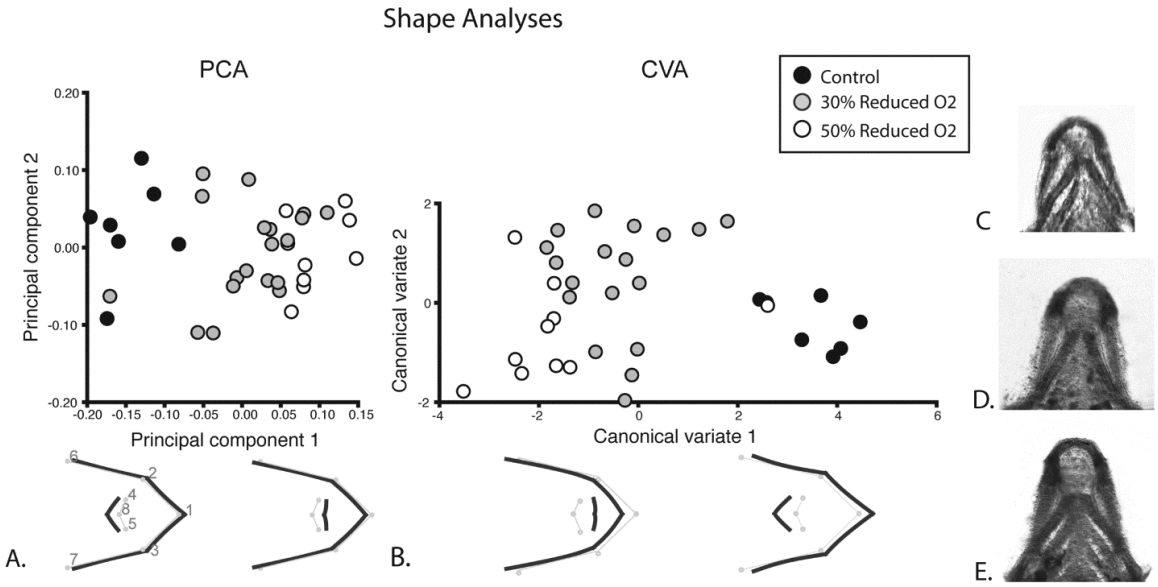


**Figure 2**: Shape analysis results.

The shapes corresponding to the first PC are also modeled in Figure 2. The wireframe at the negative end of the axis, corresponding to the control group, shows a slightly wider first arch and a more posteriorly located anterior portion of the second arch. The anterior most point of the first arch of this shape also extends beyond that of the over-all consensus shape, which is the average shape derived from all specimens included in the analysis. The wireframe corresponding to the positive end and the 50% oxygen group appears slightly 'compacted' relative to the consensus; it appears slightly narrower in all respects and the second arch appears anteriorly displaced, while the anterior most point of the first arch is located relatively closer to the second arch.

The CVA also revealed a difference in shape variation between the three groups. SImilar to the PCA scatter plot, the first CV shows three discrete clusters with the controls at one end, 50% oxygen group at the opposite end, and the 70% oxygen group in the middle. The corresponding shapes also mimic those of the PCA, largely confirming the analysis.

Neither 70% nor 50% hypoxia exposure at 24 hpf for 24 hours resulted in excess embryo mortality. However, mortality rates at three months were increased 50% and 22%, respectively. None of the treatments caused obvious vascular phenotypes, although this judgment was based solely on gross visual inspection.

## DISCUSSION

The results of this hypoxia experiment show that reduced oxygen availability changes the morphology of the anterior viscerocranium in developing zebrafish. Additionally, the extent of the phenotypic outcome depends on the severity of hypoxia exposure. These preliminary results support the hypothesis that hypoxia is one of many environmental factors capable of altering craniofacial development, specifically first and second arch-derived structures. While these shape differences could have been a result of developmental stage differences, every attempt was made to harvest specimens at the same time point. To further reduce error due to size, the shape data were subjected to a regression to minimize size effects in the sample. This would have also reduced shape differences due to developmental stage, as stage is highly correlated with size in zebrafish [19].

The data as presented in the PCA in Figure 2 reveal that the largest axis of variation in the sample separates the first and second branchial arch shapes of the three groups. The 50% oxygen group is at one extreme end of the shape spectrum and the controls at the opposite extreme. Interestingly, the 70% oxygen group spans the entire area of variation between the other two, overlapping with both groups. This indicates that while there is a dosage affect and a 50% reduction in oxygen level leads to anterior viscerocranial shapes outside that of normal (control) shape variation, a 30% reduction has widely variable results. In some animals it leads to the "extreme" phenotype, while others are within in the realm of normal variation. The CVA and permutation tests statistically confirmed the mean shape differences between the three groups. This type of result from the 70% oxygen group fits previous observations of the possible effect of hypoxia on human craniofacial anomalies; prevalence is increased, but the deleterious effects are not uniform and exhibit a range of severity [2,5]. Moreover, a similar dose-dependent effect and wide range of phenotypic response has been reported in the developing craniofacial complex of chicks following acute hypoxia exposure [4].

Both the PCA and CVA indicated similar shape deformations associated with the treatment groups. Two shape differences of note between the hypoxia treatment groups and the control group were at the anterior most ends of the first and second arch. Part of the first arch gives rise to the mandible, and therefore the posterior location of the anterior arches in the treatment groups relative to the control group could be interesting in several ways. Conclusions would depend on their relative position to the developing upper jaw. If these arches are epigenetically integrated with the developing palate, reduced oxygen during gestation could have implications on facial outgrowth. There are indications that these structures may indeed be integrated, as the shape of the adult fish lower jaw is highly correlated with the

neurocranium leading some to conclude that these craniofacial elements are under the same genetic control [20]. Additionally, many of the zebrafish mutants with craniofacial phenotypes exhibit both affected jaws and anterior neurocrania[11,21,22]. That these structures appear to be under similar genetic control makes it tempting to speculate that they might have similar reactions to environmental factors and therefore hypoxia could hinder outgrowth in both structures, not just the first branchial arch.

As mentioned above, the shape of the second branchial arch is also affected by the hypoxia exposure. The 50% reduced oxygen second arch shape is both located anteriorly and more widely angled compared to the shape associated with the control group. Altered shape of the developing arches could have implications for a relationship between hypoxia and several branchial arch syndromes. An example of one such syndrome is oculo-auriculo-vertebral syndrome (OAVS; OMIM 144210), which encompasses a wide spectrum of phenotypic variability that includes microtia, hemifacial microsomia and other mild ear malformations such as preauricular tags, and vertebral defects either singly or in combination. The etiology of OAVS is unclear, but it is thought to result from dysmorphogenesis of first and second branchial arch derivatives [23]. Interestingly, when compared to low elevation populations, isolated microtia and the expansive OAVS phenotype have been found at higher frequencies in infants born to women in the high altitude city of Quito, Ecuador [2,5].

While both hypotheses tested in this study are supported by the data, the conclusions are of limited scope. Although zebrafish are phylogenetically far removed from humans, much of our biomedical knowledge has been studied through the use of animal models. Indeed, the zebrafish larvae form the same skeletal elements as 'higher' vertebrates and the zebrafish is increasingly used to study jaw formation specifically [24]. While these results do not directly apply to mammals and humans specifically, they do provide evidentiary support that further research into this interaction between hypoxia and craniofacial development is warranted. Now that a clear phenotypic effect has been established, a natural next step would be to elucidate the biological mechanisms underlying it. Recent experimental work by Smith and colleagues [4] suggests that hypoxia-induced craniofacial malformations in chick embryos result from a combination of increased cell death and decreased cell proliferation. Zebrafish provide an excellent avenue for further investigations into the possible genetic and molecular machinations of hypoxia's effects on craniofacial development. Additional experiments testing hypotheses of gene-environment interactions involving hypoxia and different craniofacial phenotypes would provide useful insight into determining the etiology of complex and multifactorial human craniofacial malformations such as OAVS and cleft lip/palate.

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