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Effects of Temperature and Water Flow on Morphology of Astyanax mexicanus (Teleostei: Characidae)

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Effects of Temperature and Water Flow on Morphology of *Astyanax mexicanus* (Teleostei: Characidae)

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By

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1 SUMMARY

Most aquatic vertebrates are ectotherms. As a consequence, they are particularly susceptible to variation in environmental conditions during development, which can impact their phenotypes and survival. In fishes, vertebral morphology and body shape are particularly susceptible to changes in temperature and water flow regimes, two variables that are often correlated along altitudinal gradients in streams. This study addresses the impact of variation in these ecologically important environmental variables on the phenotype of the emerging model species *Astyanax mexicanus* (Teleostei: Characidae). Specifically, this study examines how variation in temperature and water flow impact vertebral number and body shape variation. Fish from seven different lab-reared crosses were pooled and subjected to four temperature treatments (20°C, 23°C, 25°C, and 28°C) for one month. They were then split into two different water flow regimens (flow and no flow) for an additional four months. A significant difference in the number of precaudal vertebrae was documented for fish reared under the different temperature treatments, with fish reared at 20°C having significantly more precaudal vertebrae than fish reared at higher temperatures. However, the number of caudal vertebrae and the total number of vertebrae did not differ significantly between temperature treatments, although the latter was only marginally non-significant. No effect of water flow treatments on vertebral number was detected, which is consistent with vertebral number being set early in development. A higher number of fused vertebrae was also documented in the 20 and 28°C temperature treatments, consistent with the breakdown of canalization mechanisms outside of the range of normal development. Body shape variation was significantly associated with body size, temperature, water flow treatments, the interaction between water flow and temperature, total vertebral
number and the ratio of precaudal to caudal vertebrae. Allometry accounted for the greatest component of body shape variation, followed somewhat surprisingly by temperature treatment. Temperature treatment was associated with variation in the upper jaw, the ectocoracoid, an increase in the size of the caudal peduncle and an increase in the dorsal fin and anal fin base lengths. Water flow treatment was associated primarily with variation in body depth. Fish subjected to the additional water flow were more streamlined than fish reared in still water, consistent with expectations from ecomorphological models. Variation in vertebral number within temperature treatments was associated mostly with body elongation, with fish having more vertebrae having more elongate bodies. Finally, the ratio of precaudal to caudal vertebrae was also significantly associated with variation in body shape, with fish having a greater proportion of precaudal vertebrae exhibiting an expansion of the abdominal region and fish having a greater proportion of caudal vertebrae exhibiting an expansion of the caudal region. The findings of this study, could apply to other aquatic species of characids, possibly including those experiencing rapid changes in their habitats. Additional studies are needed in order to more completely understand the influence of environmental factors in generating morphological variation through phenotypic plasticity. Examining how the morphological changes documented here impact other traits affecting fitness like swimming performance would provide greater insight into their functional significance.
Understanding how morphological variation arises and contributes to the diversity of animals is one of the major goals of the study of evolution. Phenotypic plasticity describes the ability of a genotype to produce a range of phenotypes in response to interactions with the environment, and enables organisms to develop distinct potential functional phenotypes across a range of environmental conditions (West-Eberhard, 2003; Moczek et al., 2011). Phenotypic plasticity is an important biological concept that has been widely studied in many different organisms, for example differences in egg production due to periods of ample larval nutrition in mosquitos (Bradshaw & Lounibos, 1977), changes in wing phenotypes due to cold environment in butterflies (Shapiro, 1977), differences in head size due to different isle locations in snakes (Keogh et al., 2005), and changes in behavior due to food availability in salmon (Taylor et al., 1996). It is extremely common in nature and is often the first form of response to any change in the conditions under which an organism develops. As such, plasticity is fundamentally important for the survival ability of organisms, as well as their ability to respond to novel environmental conditions.

Phenotypic plasticity can also be important from an evolutionary perspective because it can evolve. Phenotypic changes caused by environmental stimuli can lead to phenotypic accommodation, which is the ability of the phenotype to change in order to accommodate itself in response to a drastic change (West-Eberhard, 2003). Phenotypic accommodation may increase fitness and lead to the production of a beneficial novel trait or trait combination under particular environmental conditions (West-Eberhard, 2003). Classic examples of phenotypic accommodation often involve anomalies that occur during development. For example, West-
Eberhard (2003) describes the case of a two-legged goat born without forelegs that adopted a semi-upright posture and bipedal locomotion. As a consequence, it exhibited enlarged hind legs, changes in its muscles and bone formation in its pelvis, morphological specializations similar to those of bipedal mammals that allowed it to walk upright. Other more recent examples include experimental gene knock-outs at certain points in development, where the genetic defect is compensated by changes in behaviors or changes in the phenotype, which compensate for the non-functional gene (Greenspan, 2001).

Phenotypic plasticity can also eventually lead to genetic accommodation, a process that is typically more discrete, in which changes in the phenotype that are originally plastic and caused by environmental stimuli, eventually come under the control of genes and can be expressed as a normal part of development, without the need for the original environmental stimulus. Genetic accommodation may improve a novel phenotype by adjusting the regulation of gene expression, thus adjusting the form of the trait, by reducing disadvantageous effects of the phenotype, or by changing the conditions under which it is expressed (West-Eberhard, 2003). The classic example is the crossveinless phenotype in *Drosophila* described by Conrad Waddington (Waddington, 1956). Here, *Drosophila* pupae were subjected to a heat shock during their development. This caused the absence of a crossvein in the wings. The phenotype was triggered by the heat shock, but individuals varied in the extent to which the crossveinless phenotype was produced. In some individuals the crossvein was only interrupted or partially lost while in others it was completely lost. When this trait was subjected to selection and individuals exhibiting the most extreme loss of the crossvein were chosen as the breeders for the next generation, the phenotype increased in frequency and began being expressed without the environmental stimulus after several generations of selection. Another more recent example was described by Robinson (2013) in
fish. He compared differences in growth in freshwater and saltwater stickleback populations and found that rapid growth repeatedly evolved in freshwater compared to saltwater populations. The variation in growth was greater in the saltwater population when he compared siblings of saltwater fish that were raised in fresh water to freshwater populations. This indicated that there was a plastic response towards fast growth in the saltwater population that was not common in that environment, but was exposed and eventually selected for under low salinity conditions. These examples suggest the potential adaptive effects of phenotypic plasticity when populations are subjected to new environmental conditions and how a favorable plastic trait could eventually become integrated into the genetic machinery of populations that colonize novel environments. Thus beyond informing our understanding of how some of the phenotypic variation observed in nature is generated, studying phenotypic plasticity may provide some insight into the evolutionary potential of lineages.

Among vertebrates, fish are a particularly good group in which to study phenotypic plasticity. Fishes are the most diverse groups of vertebrates, with more than 30,000 species (Nelson, 2006). This taxonomic diversity is correlated with a great diversity of body forms and their ectothermic condition makes them particularly susceptible to the effects of environmental conditions during development. Among fishes, the Characidae are among the most species rich families, with approximately 960 to 1,200 species (Hubert & Renno, 2006), numbers that are only exceeded by the Cyprinidae, Gobiidae, and Cichlidae (Nelson, 2006). They are found from southern North America to northern Patagonia, Argentina. Characids occupy many different habitats. While most of them live in stream and rivers, some occur in ponds, lakes, and lagoons, and others can even live in underground caves (Estrada, 1999). Most of the diversity of species occurs in the Amazon, La Plata and Orinoco rivers (Hubert et al., 2006). The high diversity of
the Characidae makes them a frequent object of evolutionary and ecological studies (Sadoglu, 1956; Estrada, 1999; Jeffery, 2001; Borowsky, 2008; Jeffery, 2008; Mirande, 2010; Wilkens, 2010; Hinaux et al., 2011). However, there is still much to be learned about the potential factors that contribute to the high diversity or the broad range of ecological roles that they can occupy.

In nature, temperature and water flow vary considerably along streams and are very important in influencing the structure and morphological characteristics of fish communities. Even within streams, the range of temperatures can vary from one part of the stream to the next. This variation in conditions results in the adaptation of different species to a given range of temperatures and water flow conditions. As a consequence of this specialization, fish often function poorly outside of the range of conditions that they are adapted to (McKenzie et al., 2013). Although conditions can vary considerably within streams, there is often a pattern to the variation observed, especially in Neotropical streams originating in mountains. Temperature upstream is often lower because environmental temperatures are lower at higher altitudes, with mean temperature cooling between 10°C per kilometer (Angilletta, 2009). Water flow also tends to be higher upstream because of the greater slopes found at higher altitudes and the narrower stream widths (Hynes, 1970). Thus, these factors can be associated in natural streams and may interact to impact the phenotype of fishes inhabiting them, making them particularly relevant for understanding the sources of phenotypic variation in fish communities.

Water temperature is one of the most common environmental factors studied in relation to the phenotypic plasticity of fishes (Taning, 1952; Lindsey & Arnason, 1981; Ahn, 1999; Roy et al., 1999; Connolly, 2008; Sfakianakis et al., 2011; Grunbaum et al., 2012; Ackerly, 2013; Ramler et al., 2014). Although temperature can influence the development of multiple phenotypic characteristics including cranial morphology (Atukorala, et al. 2013), fin ray number (Taning,
1952; Mcdowall, 2003; Sfakianakis et al., 2011), overall morphology and body shape (Sfakianakis et al., 2011), general early embryonic development (Kwain, 1975; Roy et al., 1999; Sfakianakis et al., 2011; Ramler et al., 2014), and muscle development (Sfakianakis et al., 2011; Ackerly, 2013), the effect of temperature on variation of vertebral number in fishes has been an active area of research since as early as the 1800’s (Jordan, 1891; Hubbs, 1922, 1926; Gabriel, 1944; Taning, 1952; Ahn, 1999; Connolly, 2008; Sfakianakis et al., 2011; Kimura et al., 2012). Early studies described an inverse relationship between latitude and number of vertebrae (Jordan, 1891). In general, populations inhabiting cold waters have a greater number of total vertebrae than populations inhabiting warmer waters (Jordan, 1891). The influence of temperature on vertebral number was corroborated for many fish groups (e.g., Poeciliidae, Zoarcidae, Salmonidae, Fundulidae, Gasterosteidae, Pleuronectidae) (Taning, 1952).

It is unclear how important differences in vertebral number within species are for the fitness of individuals, however, at least a few studies have suggested that vertebral number variation can influence survival probabilities. For example, Swain (1992a, 1992b) found that threespine stickleback with a higher ratio of caudal to precaudal vertebrae, were better able to escape predator attacks which may be due to their increasing burst swimming performance, product of their relatively longer tail. However, the optimal ratio varied with size over a relatively small size range, highlighting the complexity of the relationship between vertebral phenotypes and swimming performance. Similarly, Brainerd and Patek (1998) studying several fish species found that the ability to bend the body was associated with the number of vertebrae and suggested that low vertebral number may reduce the ability to escape predators by inhibiting the rapid bending of the body during escape responses. A. B. Ward and Brainerd (2007) analyzed how vertebral number varies across different taxa. They found that body form is associated with
vertebral number across fishes although different groups can evolve elongate bodies associated with different modifications in number and/or length of vertebrae. They also found that there is regional specificity to this variation across lineages, meaning that the number of precaudal and caudal vertebrae can vary independently in different groups. Changes in vertebral phenotypes are typically set during the early stages of development, especially during early somite formation (Giudicelli et al., 2007). As a consequence, changes in temperature during somite formation can have different consequences on the axial morphology of fishes including on vertebral differentiation (identity), changes in the proportion of the types of different vertebrae, or changes in total vertebral number.

Water flow is another very important environmental factor that can influence the phenotypic characteristics of fishes and varies widely across ecosystems. This variation is hypothesized to affect the body shape and swimming behavior of fish. Langerhans and Reznick (2010) reviewed the effects of variation in water flow patterns on body shape, and the predictability of body shape being associated with specific environmental conditions. They hypothesized that increasing water flow regimes may lead to more streamlined bodies and increases in fin area, favoring steady swimming, whereas low water flow environments may favor a more unsteady swimming behavior and deeper bodies. Previous studies have demonstrated the effects of water flow on body shape, with fish exposed to high water flow rates tending to be more streamlined in order to reduce the drag caused by flow (Webb, 1976; Langerhans et al., 2003; Grünbaum et al., 2007; Fischer-Rousseau et al., 2010; Sfakianakis et al., 2011). For example, Pakkasmaa and Piironen (2000), used an experimental setup to determine the effects of water flow in young salmon and brown trout using artificial channels with regulated water flow simulating natural conditions. They found differences in head size,
body depth and fin size. Individuals in the fast flow regime showed streamlined bodies, larger pectoral fins, and longer heads. These differences were found after one month of treatment, suggesting the high plasticity of water flow on shape. Langerhans et al. (2003); (2007) found similar effects in natural populations of Characids, Cichlids, and Cyprinids. They compared samples, from different locations around the world and found more streamlined bodies in populations experiencing higher water velocities. For example, cyprinids in rainforests of East Africa in which populations experiencing higher water velocity exhibit more fusiform bodies. Another species of cyprinid, Cyprinella lutrensis in Central North America, also exhibited more streamlined bodies compared to their counterparts in dammed, non-flowing habitats (Domenici and Kapoor, 2010).

In nature, many environmental factors can act simultaneously on populations of fishes, influencing their phenotypes in complex ways. This phenotypic variation arising during development may affect their performance. There are some studies that also address the effects of phenotypic variation on performance of individuals, specifically on swimming performance (Webb, 1976; Swain, 1992a, 1992b; Greenwood et al., 2010; Sfakianakis et al., 2011; Ackerly, 2013; Tsuboko et al., 2014). Considering that in natural environments these factors act together to affect individuals during their development and impact the fitness of individuals across all life stages, it is reasonable to assume that their impacts can be complex and vary among lineages. However, there are not many studies that examine the combined impact of different environmental factors.

The present study seeks to examine the effects of these two ecologically important environmental factors that can co-vary in nature, temperature and water flow, on the phenotype of the emerging fish model species, Astyanax mexicanus. Astyanax mexicanus is a member of the
most diverse family of Neotropical fishes, the Characidae, and is generally similar in form to other tetras in the species-rich genera Astyanax and Bryconamericus. Using A. mexicanus as the focal species thus increases the likelihood of relevance for a lineage of Neotropical fishes that is ecologically very important. In addition, this species possess two ecomorphs, one living in surface rivers and streams, and the other living in subterranean caves (Estrada, 1999). The differences between them are large and relatively well understood. Whereas the river populations have the typical characteristics of a common tetra, the cave populations lack pigmentation and eyes as a consequence of their adaptation to cave ecosystems (Estrada, 1999). Extensive research on this species has provided important insight into the basics of eye development (Dowling et al., 2002; Wilkens, 2010; H. Hinaux et al., 2015), population genomics, (Bradic et al., 2013) neurogenesis, cave evolutionary adaptations (Gallo & Jeffery, 2012), behavior (Hoke et al., 2012), and physiology (Caballero-Hernández et al., 2015). The genome has also been sequenced and some genomic tools have been developed (Hinaux et al., 2013). The magnitude of morphological, physiological and ecological differences between the ecomorphs, the fact that these highly divergent populations can be crossed, and the availability of genomic resources make A. mexicanus an excellent model organism for genetics, development, and evolution (Borowsky, 2008a; Jeffery, 2001, 2008).

Four temperature regimes were considered for this study, 20°C, 23°C, 25°C, and 28°C. These temperatures were selected based on the optimal temperature range for this species (23°C to 25°C) and a low extreme temperature and a high extreme temperature. Two water flow treatments, one with additional turbid water movement and one without additional water movement, were also applied to simulate the influences of different flow regimes on phenotypic variability.
The main hypothesis of this study is that temperature and water flow variation during development will affect the morphology of *Astyanax mexicanus*. Specifically, I predict that temperature will significantly impact vertebral number variation in this species with fish reared at lower temperatures exhibiting more vertebrae than fish reared at intermediate and possibly higher temperatures. Temperature may also result in changes in the ratio of precaudal to caudal vertebrae and the extreme temperature treatments may result in an increase in the variability of vertebral number. I also predict that fish reared in treatments subjected to increased water flow will exhibit more streamlined bodies than fish reared in treatments without additional water flow.

This study will contribute to understanding the effects of two important environmental variables that often covary in nature on the phenotype of an emerging model system from one of the most diverse fish families on Earth. Studying the influence of both factors in the same experiment will potentially provide important novel insight into their combined effects on phenotypic variability, since most previous studies have examined the impacts of temperature and water flow separately. Interactions between variables may lead to important synergisms that are not predictable from studying the variables separately. In addition, because of correlations between environmental variables, examining variation in multiple variables simultaneously provides more biological realism to the study. By understanding how environment influences development, we may estimate how well an individual will perform under given environmental conditions or make phenotypic predictions given known environmental conditions. This study will also provide a more detailed appraisal of how the phenotype of *A. mexicanus* is specifically influenced by variation in temperature and water flow. How exactly are the vertebral column and body shape impacted when individuals develop at lower or higher temperatures or under different intensities of water flow? By improving our understanding of how environmental
variables impact the phenotypic characteristics of characids, this study will provide baseline information for future studies on the sources of phenotypic variability of this important group of fishes in nature.

3 METHODS AND ANALYSIS

3.1 FISH

One-year old individuals of the surface form of *A. mexicanus* were obtained from the Jeffery Laboratory at the University of Maryland to establish a breeding colony at DePaul. The individuals used are part of a line established in 2002 (4th generation) from wild-caught specimens collected in Rio Grande, Texas National Park. The number of adult individuals obtained was 15 males and 19 females. Sex identification was performed by the Jeffery Lab and sexes were kept separate at DePaul.

3.2 TANK SETUP

Thirty two 5.5 gallon tanks were used in this experiment. Adults were maintained in two 20 gallon tanks, separated by sex. The tanks were filled with treated tap water (pH 7, 800 µS salinity) following the protocol used by the University of Maryland’s breeding program (Parkhurst, personal communication). Water was aired using bubblers and external air pumps and 33% water changes were performed once a week. Parameters such as pH, ammonia, nitrite, nitrate, and chlorine concentrations were monitored weekly to ensure appropriate water quality. Adults were fed with commercial tetra flakes (Tetramin™) two times a day (once a day on weekends).
3.3 Breeding

In order to reduce stress, fish were kept under the same conditions as in the Jeffery Lab. *Astyanax mexicanus* specimens were maintained on a 14-hour light/10 hour dark light cycle. Before breeding, females were supplemented with a high fat content diet using egg yolk flakes (Pentair™) for 14 days following Borowsky (2008). Males did not need to be conditioned for breeding. Individual male and female fish were separated into breeding pairs in 5.5 gallon tanks with clean water at a stable temperature of 21°C on the morning of the breeding experiment. To induce breeding by natural spawning, the temperature was raised by three degrees (24°C) in the breeding tanks and monitored continuously to detect spawning. Spawning occurred around midnight in all crosses, as is typical for this species (Borowsky, 2008). A total of seven crosses were performed, from which seven were successful, each involving different pairs of adults. The eggs from the crosses were pooled and then distributed into the temperature treatments.

3.4 Temperature Treatment

Fertilized eggs were evenly distributed into four temperature treatments of 20±1°C, 23±1°C, 25±1°C, and 28±1°C. These temperatures were chosen because they are the close to relative fluctuations in temperature in a typical stream (Estrada, 1999). The 23-28°C temperature treatments were maintained using 25-watt aquarium heaters (Marineland Stealth: ETP25-25W). For the 20°C temperature treatment, eggs were incubated in a VWR Scientific Model 2015 incubator. Continuous observations of egg development were performed. Infertile and dead eggs could be easily identified by their appearance (white/turbid color and disorganized structure) and were removed (Borowsky, 2008). The observations continued until the eggs hatched, approximately 24 hours post-fertilization. However, differences in hatching were observed
between temperature treatments. The 20°C treatment took approximately 30 hours to hatch, while the 28°C treatment took 20 hours to hatch. During the early stages of development and early larval stages, specimens were maintained in a glass petri dish within a 5.5 gal tank to facilitate their handling. Once the larvae lost their yolk sack after 4 days post fertilization (Hinaux et al., 2011), they were fed with commercial brine shrimp larvae (Borowsky, 2008). At this point the petri dishes were removed to induce growth of larvae. The diet was maintained until fish grew to approximately 12 mm in total length. They were then gradually fed more finely ground versions of an adult diet until they began to take full flake food.

The temperature treatment was continued for four weeks to ensure that both somite and vertebral number were set prior to removal of the temperature treatment, since skeletal structures are still developing for approximately two weeks after hatching (Hinaux et al., 2011).

3.5 Water Flow Treatment

The water flow treatments consisted of adding a summersible water pump (VicTsing® 80 GPH) attached to one end of the tank to induce water movement and turbulence. Since the conditions in the field do not consist of a constant flow rate and are very variable, we discarded the use of linear flow. To protect the young larvae from the pump suction, a box made with plastic mesh (8.5 cm wide by 9 cm length) was placed around the pump and built so that it did not block the outlet stream. The pump was suspended 5 cm from the aquarium wall using wood dowels, allowing fish to swim freely between the pump and the tank. The water flow treatment began one month after hatching so that fry were large enough to swim in the tank without being injured by the flowing water, and was maintained for at least 16 weeks, after which time the fish were large enough to collect the morphological data.
3.6 Water Flow Estimation

To estimate the velocity of water movement in the water turbulence treatments, a positively buoyant object was placed close to the outlet tube of the water pump and released, and the time it took to get to the other side of the tank was measured. This was repeated three times per water pump and an average water velocity was calculated. This represented the measure of maximum water velocity. To assess what water velocity was like in other parts of the tank, a dissolution-based method was employed. Jokiel and Morrissey (1993) found that the rate of dissolution of solids is linearly related to the velocity of water flow until the surface area falls below about 30%. Thus, for our estimation, linearity was assumed. During the experiment, pieces of sucrose candy (Lifesavers™) were placed throughout the tank and their weight before and after a set time in the water under the flow treatment was measured following the approach generally outlined by Koehl and Alberte (1988). The percentage of weight loss was calculated based on the differences in weight. Differences in relative velocity of the water in different parts of the tank were estimated relative to the maximum velocity using the buoyant object method described above. In total, 27 pieces of candy were placed at approximately equal distance throughout the tank. These were distributed in three sets of nine. Each set of three by three candies was suspended using zip ties; each zip tie was separated by 5 cm (left, center and right), and the candies were placed at distances of 7 cm from one another along the zip tie (surface, middle and bottom). The three sets were separated by 9 cm from each other. The experiment was run for two minutes and the candy were removed and left to air dry for 24 hours before the weight was recorded. A control without the pumps with the same set up was also used. This experiment was run twice with water pumps picked at random. The maximum average velocity was 0.26 m/s in the surface center of the tank in front of the pump based on the previous
experimental run. The surface left side was estimated to run about 0.16 m/s, and the right side at 0.21 m/s. This difference may be due to the design of the pump outlet, which is tilted slightly to the right. The middle depth velocity was estimated to be 0.17 m/s in the center of the tank, 0.19 on the left side, and 0.17 m/s on the right. At the bottom of the tank, the estimated velocities were 0.19 m/s at the center, 0.19 m/s on the left side, and 0.18 m/s on the right.

3.7 VERTEBRAL COUNTS

Before collecting vertebral data, all individuals were euthanized using a solution of 500mg/L of buffered tricaine methasulfonate (MS-222, pH 7.5) in water. Fish were left in the solution for ten minutes after opercular movement ceased, then transferred to 10% formaldehyde for fixation, where they were left for at least for 24 hours. Afterward, the specimens were rinsed in water and preserved in 70% ethanol. Vertebral data were collected from X-rays of the specimens that were taken at the Field Museum of Natural History (Chicago, IL) using an AXR Hot Shot X-ray Machine (Associated X-ray Corporation). All specimens were X-rayed at 35 Kv and 4 Ma for 8 seconds. X-rays were scanned at high resolution (1,200 ppi) with an HP Scanjet G4100.

The number of precaudal, caudal, and total vertebrae were counted. Precaudal vertebrae (Pre) were defined as the first vertebrae that forms the rib cage to the last vertebrae of the end of the rib cage. This definition was modified from Kimura et al. (2012), because their method was used on medaka and did not account for specialized structures such as the Weberian apparatus or the urostyle. The four integrated vertebrae of the Weberian apparatus, a constant structure composed of 4 vertebrae, were not included in the counts. Caudal vertebrae (Cau), were defined as those vertebrae lacking ribs and having hemal spines. The urostyle was not included in the
vertebral counts (Fig. 1). Transitional vertebrae between the precaudal and caudal regions were counted as caudal vertebrae because they typically had small hemal spines and lacked ribs (Fig. 1). The total number of vertebrae and the ratio of precaudal to caudal vertebrae (Pre/Cau) were included as variables in the analysis. The presence of vertebral fusions was also taken into consideration. Fused vertebrae were defined following Fraser et al (2015) and Witten et al. (2009), a vertebrae is considered fused when the vertebral body is less regular than others, generally longer, and for the presence of supernumerary neural and hemal arches (Fig. 1). The presence or absence of fused vertebrae was recorded as was the region in which the fusion occurred. Fused vertebrae were counted as two vertebrae following Fraser et al. (2015).

A generalized-linear model was used and a model of simplification approach was taken to examine whether there were significant associations between vertebral number, temperature, water flow and the interaction between temperature and water flow (T x F), using R, version 3.2.0 (The R project for Statistical Computing). A Poisson error structure was used because this is appropriate for counts data. Rare precaudal, caudal, and total vertebral counts were pooled with neighboring counts to minimize the number of zeros in the data. After pooling, each vertebral type was divided into three count categories (Table 1). Precaudal vertebrae were divided into 12, 13, and \( \geq 14 \) vertebral count categories. Caudal vertebrae were divided into \( \leq 16, 17, \) and \( \geq 18 \) vertebral count categories. Total vertebral number was divided into \( \leq 29, 30, \) and 31. Factors in the model were tested for significance by comparing the models with and without the factor of interest (Temperature, Water flow or the Interaction), and testing whether the excluded factor accounted for a significant portion of the variance in the data. The effect of temperature and water flow treatments on the frequency of fused vertebrae was analyzed using Chi-square tests.
3.8 GEOMETRIC MORPHOMETRICS

Body shape data were collected from the preserved fish using geometric morphometric methods. Geometric morphometrics is a quantitative methodology that compares differences in shape. It is a precise and accurate description that relies on powerful statistical analysis and serves as an mathematical and visual tool (Zelditch et al., 2012). Fish were straightened (if necessary) using #000 insect pins and photographed with a Nikon Coolpix P500 digital camera. Fourteen anatomical landmarks were used on each specimen to quantify body shape variation in two dimensions (Fig. 2). To better visualize the landmarks, #000 insect pins were used to indicate the location of some of the anatomical structures in a lateral view. The images were digitized using TPSDIG, version 2.17 (Rohlf 2010). The landmark points were aligned using Procrustes superimposition in MorphoJ (Klingenberg, 2011) to eliminate variation related to the location, rotation and size of the specimens.

It is known that size affects body shape in vertebrates. A two way factorial ANOVA was used to examine if the differences in body size between treatments were significant. The analysis was conducted using the mean centroid size of each tank. The analysis indicated significant variation in size among tanks (Table 2a) across temperature treatments, so linear regression of body shape on centroid size was used to correct the effects of size variation among tanks on body shape (Zelditch et al., 2012). The linear regression produces residuals around the predicted values that are uncorrelated with the independent variable, centroid size, and thus the residuals can be used to correct for the effects of allometry. Body size did not differ significantly between the water flow treatments.

To examine the influence of vertebral phenotypes on body shape, a MANCOVA analysis was performed with centroid size included as a covariate to predict the influence of temperature
treatment, water flow, the interaction of temperature and water flow, total vertebral number, and the ratio of precaudal to caudal vertebrae on body shape in TPSREGR, version 1.36 (Rohlf, 2009). The significance of each factor was evaluated by running the complete model, then running the model leaving out the variable of interest, and testing whether the difference between the residual sums of squares matrices is significant. To evaluate the relative importance of each factor, all the variables were scaled using the partial $\eta^2$, which evaluates the explanatory ability of a factor relative to unexplained variation (Langerhans and DeWitt, 2004). Partial $\eta^2$ s takes into account differences in the number of states of the independent variables, correcting for a bias toward overestimation of explained partial variance due to additional levels of the variables (temperature and the interaction of temperature and water flow, in this experiment). This allows all the factors to be in a common scale in order to assess their relative importance in the model.

To visualize the variation in body shape, a principal component analysis (PCA) on the body shape covariance matrix and canonical variate analysis (CVA) were conducted. PCA shows the variation of body shape and their distribution in a simplified way. This analysis is exploratory and is useful for a general visualization of the distribution of the data and for identification in the major patterns of variation in the data. CVA is similar to PCA in that it is a multivariate method of analysis that is intended to simplify complex patterns of variation, but it differs in that it is designed to specifically find the axes in multivariate space that best distinguish among sets of predefined groups. CVA uses patterns of within-group variation to scale the axes in a new set of coordinates. The CV distances indicate which groups are most effectively different. Both methods produce new set of variables that are linear combinations of the original variables (Zelditch et al., 2012). The analyses were conducted in MorphoJ version 1.06d (Klingenberg, 2011). The morphometric analysis was conducted using individual data (n=313) and this is the
data described in the results section. However, to better visualize the pattern of body shape variation in the plots, all the analysis were repeated using the tank means. The tank means are shown in the figures while the tables show the results of the analyses conducted on individuals. This approach reduced clutter of morphological variation in the PCA and CVA plots. A single PCA was conducted to explore patterns of variation among the samples based on temperature and water flow treatments. Three CVAs were conducted, one accounting for both environmental variables (temperature and water flow) in a single analysis, a second one to specifically focus on differences between flow treatments since I expected water flow to be the primary factor impacting body shape variation based on previous studies, and a third accounting for differences in the total vertebral number within temperature treatments to examine how vertebral number and body shape are associated within treatments. To quantify differences among groups, the Mahalanobis distance was calculated. This distance is interpreted approximately as the distance measured between groups scaled by the within-group standard deviation (Zelditch et al., 2012).

For water flow, a discriminant function analysis (DFA) was also conducted to further examine the variation of body shape associated with differences in water flow regimes. DFA and CVA generally produce similar results, since both try to find the axes of variation best discriminating between predefined groups while minimizing within group variation. However, when there are only two groups to compare, DFA provides more information, since it also provides classification statistics of individuals based solely on their body shape. For each CVA and the DFA, permutations were performed using 10,000 permutations to test for significance.
4 RESULTS

4.1 VERTEBRAL COUNTS

The frequency of fused vertebrae differed significantly between temperature treatments but not between flow treatments (Table 3). There was a higher frequency of individuals with fused vertebrae at the extreme temperatures. The temperature treatment with the greatest frequency of fused vertebrae was the 28°C treatment (48% of 76 individuals), followed by the 20°C treatment (42% of 77 individuals). The treatment with the least fused vertebrae was the 23°C treatment (16% of 80 individuals), followed by the 25°C treatment (25% of 80 individuals) (Fig. 3).

Vertebral number differences were significant for precaudal vertebrae in association only with temperature treatments (p= 0.001), but not for water flow or the interaction between temperature and water flow. The number of caudal vertebrae and total vertebrae did not differ significantly between temperature treatments, flow treatments or the interaction between the two variables (F x T). However total vertebral number was only marginally non-significant for temperature (p= 0.09).

The total number of vertebrae ranged from 27 to 32 vertebrae. The most frequent total vertebral count across treatments was 31 (51.4%), followed by 30 (33.2%). Only one individual had 27 vertebrae, and 8.6% of individuals had 32 vertebrae. The temperature treatment with the highest mean total vertebral count was 20°C (30.82 for the no water flow and 30.79 for the water flow treatment) and the treatment with the lowest mean number of total vertebrae was 23°C (30.43 for the no flow and 30.53 for the flow treatment). The 25°C and 28°C temperature
treatments were generally comparable in total vertebral number and intermediate between the 20°C and 23°C treatments (Fig. 4a).

The number of precaudal vertebrae ranged from 12 to 14, with the most common count being 13 (80% of all individuals). Individuals from the temperature treatments located within the range of natural populations (23°C and 25°C) had similar means for the number of precaudal vertebrae (13.00 – 13.08), the 20°C treatment had the highest precaudal mean (13.22) and the 28°C had the lowest mean for the number of precaudal vertebrae (12.96), consistent with a declining trend in the number of precaudal vertebrae with increasing temperature.

Caudal vertebral counts were more variable than precaudal counts and ranged from 14 to 19. The most common number of caudal vertebrae across temperature treatments was 18 (47%), followed by 17 (36%). Only one individual had 14 vertebrae and 8% of individuals had 19. The 20°C and the 25°C treatments had similar means for the number of caudal vertebrae, 17.58 and 17.56, respectively. The temperature treatment with the lowest mean number of caudal vertebrae was the 23°C treatment with a mean of 17.41, while the treatment with the highest mean number of caudal vertebrae was the 28°C treatment with a mean of 17.65 (Fig. 5).

The numbers of precaudal and caudal vertebrae were negatively correlated across treatments (r= -0.32, n=313, P<0.01), suggesting that additions or losses of vertebrae in one body region were often associated with the transformation of vertebral identities rather than with an increase or decrease in total vertebral number. This relationship held within temperature treatments for the 20°C and 23°C treatments (r= -0.54 and -0.48; P<0.01), however, it was not significant for the 25°C (r= -0.17, P=0.127) or the 28°C treatment (r= -0.16, P=0.158). Across treatments, the ratio of precaudal to caudal vertebrae exhibited a declining trend such that fish in the 20°C treatment had high precaudal/caudal ratios while fish in the 28°C treatment had the
lowest precaudal/caudal ratios. This translates to fish in the 20°C treatment being biased towards having a greater proportion of precaudal vertebrae and fish in the 28°C being biased towards caudal vertebrae, with the 23°C and 25°C treatment fish being intermediate.

4.2 **GENERAL BODY SHAPE VARIATION**

The MANCOVA analysis indicated that there were significant effects of all the factors in the regression model on body shape variation. Wilk’s partial η² was used to estimate the magnitude of the effect of each variable (Langerhans & Dewitt, 2004). Centroid size was the most important variable, accounting for approximately 35% of the partial variance in the data, and indicating that there was significant allometric variation in body shape. The second most important variable was temperature (24% of the variance), followed by water flow (22% of the variance), vertebral ratio (17% of the variance), the total vertebral number (15% of the variance), and the interaction between flow and temperature (11%) (Table 4).

Body shape variation attributable to allometry was associated mainly with the head size and caudal regions (Fig. 6). Predicted shapes for individuals with small centroid sizes (Fig. 6a) indicated relatively larger heads and larger eyes, a reduction in the length of the base of the anal fin, a longer caudal peduncle, and a more slender body. Shape predictions for larger individuals indicated relatively, smaller heads, smaller eyes, a greater length of the base of the anal fin, greater depth and a shortening of the caudal peduncle, and a relatively deeper body (Fig. 6b).

The PCA of the size-corrected body shape data did not show a strong pattern of segregation of specimens based on the treatments, suggesting that the effects of the temperature and water flow treatments on body shape variation in this experiment were relatively subtle. However, some patterns were apparent. The first PC explained 21.59% and PC2 explained
16.47% variation of the body shape. There seemed to be some clustering by temperature treatment, with most tank means from the 20°C treatment clustering towards the top left portion of the plot and tank means for the 28°C treatment clustering towards the lower right portion of the space. Tank means from the 23°C and 25°C treatments were scattered and generally intermediate to the extreme temperature treatments. PCs 3, 4, 5, and 6 accounted for 10.76, 6.87, 6.54, and 5.19% of the variation in body shape for a cumulative total of 67.39% of the variance in body shape explained by these axes (Fig. 7). Images depicting the predicted body shapes associated with variation along the first two PC axes allowed interpretation of the major patterns of body shape variation. The body shape associated with the negative end of PC1 exhibited a reduction on body depth, an increase in the length of the base of the anal fin, and an increase in the length of the caudal peduncle. There was also a slight a reduction in the size of the head, with the mouth and the eye were displaced slightly downward (Fig. 7a). PC1 on the positive end was associated with a deeper body, a shorter caudal peduncle, a small increase in the depth of the head, and a lower position of the base of the pectoral fin. The eye also seemed to be displaced upward (Fig. 7b). The negative end of PC2 was associated mainly with a decrease in size of the precaudal area (abdomen), a relatively large increase in the base of the anal fin, and an anterior displacement in the position of the base of the pelvic fin (Fig. 7c). The positive end of PC2 was associated with an increase in the size of the precaudal area, a reduction in the length of the base of the anal fin base, and a posterior displacement of the base of the pelvic fin (Fig. 7d).

4.3 BODY SHAPE VARIATION ASSOCIATED WITH TEMPERATURE TREATMENTS

The general CVA, based on the individual data, accounting for differences between both types of treatments (temperature and water flow) indicated a clear separation among groups. The first CV explained 37.11% of the variance in body shape and the pattern seems to be related to
temperature, specifically differences in body shape between the 20°C treatment and the other groups. The second CV explained 23.8% of the variance, and appeared to be associated primarily with water flow based on how the sample means segregate. However it was also associated with temperature, specifically segregating samples associated with the 23°C treatment and the other groups (Fig. 9). The total variation explained a total of 60.99% between the two CVs (Fig. 8).

The shape consensus for CV1 was associated with differences in the head, the dorsal fin and anal fin base lengths. The negative end of CV1 was associated with an increase in length of the anal fin, a reduction of the length of the base of the dorsal fin, and a dorsal displacement of the upper jaw, increasing the area of the mouth (Fig. 8a). The positive extreme of CV1 was associated with the upper jaw moving ventrally and the ectocoracoid moving dorsally, indicating a possible reduction of the head area, a dorsal displacement in the position of base of the pectoral fin, an increase in the length of the base of the dorsal fin, and an increase of the distance between the adipose fin landmark and the origin of the caudal fin membrane in the dorsal midline that resulted in a deviation of the base of the caudal fin landmarks to a more ventral position (Fig. 8b.). There was also a reduction of body depth as indicated by the movement of the landmark associated with the anterior end of the anal fin. CV2, which as described above tended to be associated with the water flow treatments, pointed mainly to differences in body depth. A generally more streamlined body is predicted for samples subjected to the water flow treatment and a deeper body is predicted for samples reared under the no-flow treatment (Figs. 8c and 8d).

The Mahalanobis distances (M) among treatments were mostly significant among the groups (Table 5). Only three pairs of treatment means did not differ significantly for the permutation tests. These were the means for the 25°C no-flow and 23°C no-flow treatments, the 25°C flow and no-flow treatments, and the 28°C flow and 25°C flow treatments. These groups
tended to be close in the CV plot (Fig. 8). The greatest Mahalanobis distance was between the extreme temperatures groups, 28°C flow and 20°C no-flow. The lowest distance was between the middle temperatures 23°C and 25°C, specifically between no-flow treatments. For water flow regimes (within temperatures), the distances between groups were 60% greater than flow relatively to no-flow treatments, when the greatest distance was scaled to 100%.

4.4 BODY SHAPE VARIATION ASSOCIATED WITH WATER FLOW TREATMENTS

To better examine differences between water flow treatments, a separate CVA for individual data was conducted for this variable. The CVA for water flow had a single CV axis, it showed a clear separation between the groups (Fig. 9). The negative end of the CV axis grouped the individuals from the no-flow treatment and the positive end, the ones from the water flow treatment. The consensus shapes differed mainly in body depth, as was the case in the combined analysis of temperature and water flow treatments. The body depth increase in the pelvic fin region was particularly pronounced. The no-flow shape also showed a reduction in the distance of the landmarks of the caudal peduncle. The predicted shape for individuals in the flow treatment indicated a reduction in body depth in the same region, while the length of the base of the anal fin and the caudal peduncle increased (Fig. 9a and 9b). The DFA conducted using all specimens also indicated a relatively strong and statistically significant divergence in body shape based on the water flow treatments (p<0.001). Of the 313 specimens in the experiment, 72% were correctly classified to the water flow treatment to which they were subjected based on body shape alone (Table 6).
4.5 THE ASSOCIATION BETWEEN BODY SHAPE AND VERTEBRAL PHENOTYPES

The MANCOVA analysis indicated that total vertebral number and the ratio of precaudal to caudal vertebrae had a significant effect on body shape variation. The predicted shape differences based on these variables were generated using tpsRegr ver. 1.41 (Fig. 10). The main change in body shape observed associated with variation in total vertebral number is related to body elongation (Fig. 10a, 10b). Individuals with low vertebral number had shorter bodies, whereas relative length increased in individuals with higher vertebral numbers. The head region seems to be affected as well. For individuals with lower vertebral number, the position of the upper jaw moves dorsally, as does the position of the eye, the head declined in size, the upper jaw moved ventrally and the size of the eye declined. The ratio of precaudal to caudal vertebrae is associated primarily with body shape variation along the anterior – posterior axis, especially the relative size of the abdominal and caudal regions. Individuals with greater proportions of caudal vertebrae (Fig. 10c) exhibit an expansion of the caudal region of the body between the posterior end of the dorsal fin and the adipose fin, an expansion of the base of the anal fin and a deeper caudal peduncle. The shape predicted for individuals with greater proportions of precaudal vertebrae (Fig. 10d) was associated with an expansion in the abdominal region of the body between the head and the dorsal fins, an expansion of the area between the base of the pelvic fin and the ectocoracoid, and a relative increase in the size of the head, as evidenced by the increase in the distance between the mandible and the ectocoracoid. In terms of the Mahalanobis distances, within temperatures, the distances were significant for certain vertebral phenotypes. For the 20°C treatment, there were not significant distances between fish with 29 vs 30, 31, and 32 total vertebrae (Table 7a). For the 23°C treatment, the vertebral phenotypes that were not significant were specimens with 28 vs. 29 and 28 vs. 32 vertebrae (Table 7b). For the
25°C treatment, the significant distances were between specimens with 31 vertebrae the rest of the specimens (27, 29, 30, and 31) (Table 7c). For the 28°C treatment, all distances were significant, except specimens with 28 vs. 29 and 30 vs. 31 vertebrae (Table 7d).

5 Discussion

Phenotypic plasticity is the first response of an organism to changes in the environment and can be very important for allowing organisms to persist under novel environmental conditions, and eventually adapt genetically (West-Eberhard, 1989). Environmental factors can have multiple effects during the development of organisms. Temperature and water flow are particularly important environmental factors that can vary substantially over relatively small geographic distances in some parts of the world, like in Neotropical streams originating in mountains. Fish were chosen for this study because of their tremendous biological diversity and because as ectotherms, both temperature and water flow variation can significantly affect their phenotype during development and at later stages (Hubbs, 1922, 1926; Gabriel, 1944; Kwain, 1975; Lindsey et al., 1981; Wimberger, 1992; Johnston, 1993; Ahn, 1999; Roy et al., 1999; West-Eberhard, 2003; O'reilly & Horn, 2004; Kouttouki et al., 2006; A. B. Ward et al., 2007; Koumoundouros et al., 2009).

The purpose of this project was to study the effects of changes in environmental conditions during development in the emerging model system A. mexicanus. The primary aim was to determine if temperature and water flow variation during development affected the general morphology of A. mexicanus, and if so, how. For the temperature treatments, it was hypothesized that vertebral phenotypes (presence of fused vertebrae, total vertebral number and the ratio of precaudal to caudal vertebrae) would be affected primarily by temperature. It was expected that a
higher number of fused vertebrae would be present in the extreme temperatures 20°C and 28°C because of the impact that extreme environmental conditions are known to have on developmental stability. It was also expected that individuals reared at low temperatures would have higher vertebral counts, as has been found in many studies following Jordan’s (1891) discovery of the relationship between temperature and vertebral number. For the water flow treatment, it was hypothesized that individuals subjected to additional water flow would have more streamlined bodies compared to individuals that were reared in standing water based on insights from previous ecomorphological studies. We also hypothesized that variation in temperature might impact body shape and that variation in vertebral phenotypes might be associated with variation in body shape within treatments.

5.1 VERTEBRAL PHENOTYPES

Vertebral phenotypes were significantly impacted by variation in temperature during development. The frequency of fused vertebrae was significantly higher in the extreme temperature treatments. More than 40% of individuals of the 20°C and 28°C had at least one fused vertebra. The number of total vertebrae did not differ significantly among the temperature treatments (p= 0.09) although the test was only marginally non-significant and their seemed to be some grouping of samples based on temperature treatments. However, there was a significant effect of temperature on the number of precaudal vertebrae. Individuals from the 20°C treatment had more precaudal vertebrae than fish subjected to the other temperature treatments. Individuals reared at 28°C had a lower mean number of precaudal vertebrae. For caudal vertebrae, individuals reared at 28°C presented a higher number compared to the other temperatures. Temperature also had an effect on body shape in addition to the water flow effect. These findings highlight the different effects of temperature on various traits and how something as simple as
temperature variation can lead to notable morphological changes in the development of individuals.

Even though determining the exact point during development at which vertebral anomalies form was not the main focus of my study, previous studies suggest a possible explanation for the increase in the frequency of fused vertebrae under the extreme temperature treatments. First, from an evolutionary perspective, the variation in the frequency of vertebral fusions across temperature treatments could be explained by canalization theory. Canalization is a theoretical model that suggests the occurrence of nonlinear effects between environmental factors and phenotypes. For example, within the temperature range typically experienced by the species, the average effects of temperature on the phenotype will likely be small. Individual variation in the temperatures experienced will have little effect on phenotypic variation because development is canalized towards particular phenotypes under optimal temperature conditions. Beyond the temperature range that a species typically encounters, the developmental effects are more pronounced and organisms may experience developmental instability, unmasking hidden genetic variation within the population. Sometimes, this variation can be detrimental to fitness. For example Fraser, et al. (2015) demonstrated that increasing egg incubation temperature results in a higher prevalence of vertebral deformities in salmon, where they found nearly 23% of fish had at least one deformity present, although they included several deformities, not just vertebral fusions. Sometimes the variation can be neutral with respect to fitness or rarely even adaptive. Nonetheless, individual responses to variation in temperature will translate to higher phenotypic variation (Gibson & Wagner, 2000; Ramler et al., 2014). Thus, changes in the environment could influence the evolutionary course of organisms, resulting in an increase in phenotypic variation that could accelerate the population’s response to natural selection. In the context of
our study, we observed a higher count of fused vertebrae in both extreme temperature treatments, suggesting developmental instability under these conditions. The lower number of individuals with fused vertebrae at intermediate temperatures, may suggest some phenotypical canalization of vertebral phenotypes at temperatures of 23°C to 25°C, which are close to the optimal described for the species.

How does temperature impact the number or identity of vertebrae in fishes? The answer is likely complex and may vary depending on the nature and timing of environmental stimuli. Connolly (2008) reported the effects of heat shocks on the axial skeleton of zebrafish and suggested that embryos are more susceptible to environmental changes during some developmental stages than others. Specifically for the total number of vertebrae, Connolly (2008) reported a decrease in total vertebral number following a heat shock at the 12 somite stage. This study also found that 28-71% of individuals submitted to heat shock present an array of anomalies, including bifurcating ribs, hemi-vertebrae and shrunken centra.

Alterations in the timing of somite formation, perhaps affecting the cell cycle, may be at play. The effects of temperature seem to be affecting cell replication (Connolly, 2008). Somites in zebrafish work as a clock, and they are formed in specific time intervals (around 30 min per somite), suggesting a mechanism were cell migration, cell signaling, and cell differentiation are crucial for the correct formation of the structures (Schroter et al., 2008). Somite formation is directed by the wavefront of the Delta/Notch signaling pathway, with boundaries being set by patterns of expression of fibroblast growth factor (FGF) in the mesoderm of the embryo (Ward & Metha, 2010). Gomez and Pourquié (2009) found that somite number is controlled by the relationship of the speed of axial elongation and somite formation. The exact mechanism that may lead to higher number of somites, and thus a higher number of vertebrae, is still unclear.
However, there are two general hypotheses that may explain this result. The first one states that the time for each somite to form decreases while the overall time of somatogenesis is the same. This would result in a higher number of smaller somites. The second hypothesis states that maintaining the posterior growth of the embryo will result in more, similar sized somites. The final length of the mesoderm would not differ between temperatures (Gomez et al., 2009). Individuals at higher temperatures would develop longer somites, thus, fewer vertebrae, than individuals at lower temperatures (A. B. Ward et al., 2007; A. B. Ward & Mehta, 2010). The results of these studies suggest the potential mechanism in which temperature alters the process of somite formation and eventually the axial skeleton. Ward (2007) also suggests that shifts in Hox gene expression boundaries may be associated with increases in the number of precaudal vertebrae and that longer periods of somatogenesis in the tail may be associated with an increase in the number of caudal vertebrae. They also suggest that increasing the speed of the somatogenesis wavefront may be associated with the elongation of somites.

The general effect, identified by Jordan (1891), that lower temperatures increase the number of vertebrae is observed in this study. Although total vertebral number did not differ significantly among the temperature treatments, the observed pattern of variation was consistent with this effect and the number of precaudal vertebrae did differ significantly among treatments in a manner consistent with Jordan’s Rule. Since Jordan’s proposal, there have been many studies that address the impact of temperature on the axial skeleton. Hubbs (1922) compared the effects of temperature on the number of vertebrae in two types of fish that reproduce at different points of the year. Cyprinids developed at colder temperatures and Centrarchids at higher temperatures. Two generations spawned during the period of the study. He found that the number of vertebrae was correlated with temperature in both fishes. Fish developing at lower
temperatures, had more vertebrae than fish developing at higher temperatures. Gabriel (1944) studied variation of meristic characteristics, i.e., vertebrae number and rays in Fundulus heteroclitus, at three different temperatures using siblings. He also found an association between higher temperatures producing low vertebral counts and lower temperatures producing higher counts. Furthermore, he reported that siblings form parents with higher vertebral counts had higher vertebral numbers and siblings from parents with lower vertebral counts had less vertebrae, indicating that variation in vertebral number in this species also has a genetic component to it. Taning (1952) conducted experiments on sea trout and determined the most sensitive period during development for impacting vertebral development is around the start of the development of the eye. During this period, changes in temperatures of ±3°C can produce an average difference of 1.5 vertebrae. Fowler (1970) found that the curve for individual vertebral number plotted against temperature in a single generation has a U-shape. This curve shape was also observed in my study. There are many other studies that have described similar results (Lindsey et al., 1981; Ahn, 1999; O'reilly et al., 2004; Georgakopoulou et al., 2007; Kimura et al., 2012); the number of vertebrae is affected by temperature and colder temperatures generally produce higher numbers of vertebrae. In a similar study to that conducted here, Ackerly (2013), found a significant effect of temperature on the number of caudal vertebrae in the zebrafish (Danio rerio), and similar to our results, the total number of vertebrae in their experiment was also not significant. A. B. Ward et al. (2007) suggest that in the Ostariophysi, the superorder to which characids belong (and also the zebrafish), there tends to be greater variation in the number of vertebrae in the caudal region. Although I did find greater variation across all specimens in the number of caudal vertebrae present, significant differences among temperature treatments were only documented for the precaudal region. However, only individuals from the 20°C presented a
higher number of precaudal vertebrae. The difference between this study and that of Ackerly (2013) suggests that the impact of temperature on the number of precaudal and caudal vertebrae, as well as the ratio of precaudal to caudal vertebrae, is complex and can differ substantially in different types of fish.

5.2 Effects of Temperature on Body Shape

Temperature had a significant impact on body shape. In our model, temperature ranked second only behind allometry as a contributor for explaining body shape variation. This was confirmed by the distribution of sample means in the CVA and the significant divergence among samples based on temperature treatments. This result was somewhat surprising since the expectation was that the water flow treatments would have the largest impact on body shape variation based on previous ecomorphological work and that temperature variation would primarily impact vertebral phenotypes. Nonetheless, impacts of temperature on body shape variation have been reported previously. Georgakopoulou et al. (2007) reported that sea bass larvae that was reared at two different temperatures (15°C and 20°C) differed in meristic characteristics and shape, with development at lower temperature resulting in a more slender body, a deeper post cranial area, and lower positioning of the paired fins. Another recent study by Georga (2010) on zebrafish found that the main structures affected by temperature among fish reared at 22°C, 28°C, and 32°C were the dorsal, anal and caudal fins, as well as the gill cover and lower jaw form. In my study, the main structures that were impacted were an increasing distance between the upper jaw and the ectocoracoid, an increase in the length of the dorsal fin base, and a deviation of the base of the caudal fin (Fig. 8). The results of my study are similar to those found by Georga (2010) and Georgakopoulou et al. (2007) in the positioning of the paired fins and cranial shape. This changes in shape could be attributed to differential effects of
temperature, on the growth rate of tissues and organs (Lindsey et al., 1981). Other effects like alterations of muscle development may be contributing to the change in shape (Johnston, 1993). In zebrafish, the number of muscle fibers depends on the thermal conditions during early development. This effect may be present in our model system as well, explaining some of the effect of temperature on shape.

5.3 Effects of Water Flow on Body Shape

The results of this study follow the general trend of others that predict a more streamlined shape for fish reared under stronger water flow conditions (Pakkasmaa et al., 2000; Langerhans et al., 2003; Grünbaum et al., 2007; Langerhans et al., 2007; R. Langerhans, 2008; Domenici & Kapoor, 2010; Fischer-Rousseau et al., 2010). The main effect in this study was on body depth, especially an expansion of the body in the abdominal region associated with the landmark located on the pelvic fin. The size the fins and associated structures were also affected, with an expansion of the caudal peduncle and the anal fin base being especially pronounced. These results of shape were obtained from the CVA first and confirmed in the DFA. The discriminant analysis showed that the differences in shapes predicted by water flow were different enough to correctly classify 70% of the individuals. This result is similar to that obtained by Langerhans (2003), based on differences in body shape of characids and cichlids in lagoon vs. stream channel habitats. In both species studied, they observed an anterior shift in maximum body depth (fusiform shape) in channels population relative to lagoons. This study is important because it shows that the results of our laboratory experiment are similar to the effects of water flow observed in natural populations. Another study by Langerhans (2007) found significant differences in the form of the caudal fin. Although in our study we did not measure fin area, the distances between the landmarks placed at the base of the caudal fin and the anterior portion of
the caudal peduncle varied among individuals reared under the different water flow treatments, suggesting a change in the size of the caudal fin. These changes support the general notion of a streamlining and higher caudal fin ratio compared to the low flow habitats (Domenici et al., 2010).

Locomotor abilities are presumably under strong selection pressures, since all activities of fish are highly dependent on effective movement and aquatic environments are energetically demanding to move in. This pressure may vary by habitat. Many environments contain habitats with varying water velocities, and fishes are known to have distinct morphologies between lotic and lentic habitats (Pakkasmaa et al., 2000; Brinsmead & Fox, 2002), including a more streamlined body to reduce drag, thus reducing the energy requirements for the individuals to maintain their position when swimming against strong currents. The results of our study suggest that water flow drives phenotypic variation in a specific direction that is consistent with that observed in other studies, including those pertaining to fish in nature. Langerhans (2008) suggested that this variation in body shape resulting from variation in water flow regimes is potentially predictable between and within species of fish. The effect of water flow on body shape would be an effect that is additional to the shape variation attributable to that of the temperature treatments described above. This is important to consider in our results because it suggests that certain environmental factors may have specific effects on body shape, but as a whole, the different factors act together to influence individual variation in different proportions.

5.4 ASSOCIATION BETWEEN VERTEBRAL NUMBER AND BODY SHAPE

There was a significant association between body shape and vertebral number and the proportion of precaudal and caudal vertebrae. The distribution of individuals in the CV space
was similar across temperatures. The analysis of body shape variation associated with vertebral number was conducted separately for each temperature treatment in order to account for changes in shape independent of the impact of temperature on body shape, as well as changes in body shape that were specific to certain temperatures (see supplemental figures 4 - 7). The effect of total vertebral number was mainly associated with body elongation and the main effect of vertebral ratio was in variation associated with specific body regions, e.g., larger distances between landmarks corresponding to the base of the dorsal fin, a longer base of the anal fin, and a reduction in distance between the anterior end of the anal fin and the base of the pelvic fin for fish with a greater proportion of caudal vertebrae. The precaudal extreme was associated with an increase in distance between the pelvic fin and the ectocoracoid, plus a deeper head region relative to the caudal extreme. That is, an increase in the proportion of precaudal vertebrae was associated with an increase in the size of the abdominal region of the body, while an increase in the proportion of caudal vertebrae was associated with an expansion of the caudal region of the body, indicating that body form correlates with variation in the vertebral column even within temperature treatments.

This association between vertebral number and body shape has been documented in other studies. Aguirre et al. (2014) studied the effects of total vertebral number and vertebral ratio across several natural populations of threespine stickleback that corresponded to different types of ecomorphs (anadromous, limnetic and benthic). The variation in vertebral phenotypes suggested a significant association between vertebral number and body elongation; limnetics possessing more elongate bodies had a greater number of vertebrae. Moreover, the increase in vertebral number was primarily associated with an increase in the number of caudal vertebrae, suggesting body region specificity. Other studies (Ahn, 1999; A. B. Ward et al., 2007) also
associate increases in vertebral number with body elongation, supporting our findings, since a higher number of vertebrae was associated with more elongate bodies in this study.

Finally, the negative correlation between the number of precaudal and caudal vertebrae suggests that changes in the number of precaudal or caudal vertebrae are often associated with changes in the regionalization of these vertebral types. Precaudal and caudal vertebrae are in different regions of the body and are likely subjected to different functional demands. Changes in the proportion of these types of vertebrae are associated with changes in external body form and may have important consequences for functional performance irrespective of differences in total vertebral number. Previous studies have highlighted the importance of the proportion of precaudal to caudal vertebrae. For example, Swain (1992) found that in threespine stickleback, the ratio of precaudal to caudal vertebrae changed with size, going from a higher value (more precaudal vertebrae) to a lower value (more caudal) as body size of the population increased. This change in vertebral ratio was consistent with patterns of selection in predation experiments (Swain, 1992b), suggesting that different ratios of precaudal to caudal vertebrae were under selection based on the probability of escaping predator attacks. However, the optimal ratio varied with size over a relatively small size range, highlighting the complexity of the relationship between vertebral phenotypes and swimming performance. In any case, this example indicates that the ratio of precaudal to caudal vertebrae matters functionally and can be subjected to natural selection.

5.5 LIMITATIONS

Although this study uncovered several important results that will lay a foundation for future studies of related topics both in the lab and in nature, there are some important limitations
that must be acknowledged. This study was conducted for a single generation from one population, making it unclear how general the results are. Another important limitation is that the parental strains are from a 4th generation lab raised population. This may imply that the phenotypic variability may be reduced or differ as fish have “adapted” to conditions in the lab and thus not be representative of natural populations. Ideally, a better approach would be to study a colony from a natural environment and compare the conditions of the environment to those reared in the lab. However, our findings seem to be consistent with the results of other studies that used wild populations. Finally, differences in the total number of individuals in the different tanks may affect their grow rate for portions of the study. Individuals form the 20°C treatment grew at lower densities for part of the experiment due to greater mortality of eggs in this treatment. As a consequence, they grew the largest despite growing at a slower rate for the first month of development. As the experiment proceeded, specimens from the other temperature treatments were culled so that fish were growing under approximately equal densities after culling. Nonetheless differences in density for part of the experiment likely impacted growth rates of individuals under different temperature treatments. It is known that the shape of individuals is affected by their size. However, this effect was taken into consideration by applying a size correction method. Additionally, a discriminant function analysis between tanks within temperature treatments was conducted to check for significant differences in the shape of individuals attributable to density or other tank specific effects (data not shown). This analysis did not detect significant differences among tanks, indicating that even if there was a small tank effect, it was not significant enough to alter the results of the other factors.
5.6 IMPLICATIONS OF THE STUDY

Historically, phenotypic plasticity has often been considered developmental noise or just a source of random variation within individuals. This is not the case anymore. At an individual level, the different types of plastic responses may produce a set of individuals varying in fitness. If the conditions are met, changes in the body shape or in the axial skeleton caused by variation in environmental factors may be strong enough to have implications for the ability of individuals to perform properly in their environments. Ultimately, this may have evolutionary implications. It is important to consider that in order for a plastic response to be selected for, the environmental condition(s) that are responsible for that plastic phenotype need to be durable enough to affect multiple generations and there need to be heritable differences among individuals in the propensity to exhibit the plastic response. Only then, may there be a shift of the selective response in that particular environment. The wide range of responses observed by plastic traits, may offer the raw material for selection and thus, specialization in a given environment.

The formation of new traits is associated with the interaction between genotype and environment. Phenotypic plasticity, is common in constantly changing environments, and as such provides an important source of phenotypic variation in novel environments. In addition, depending on the nature of the population and its evolutionary history, a plastic response to a change in the environment may cause a shift in the phenotypic properties of many individuals simultaneously, resulting in similar phenotypic characteristics among many individuals in response to the novel stimulus. Novel mutations will affect one individual or a small group of relatives unless the mutation increases in frequency substantially. Studying plasticity responses in more detail may help us to understand the point during development where new traits arise,
and how they can arise. During the developmental process, the position and timing of cell activity is crucial. If the environment is affecting the way in which cells move or affects the timing of expression of certain proteins, the developmental choreography is going to be affected. These effects can accumulate and may eventually be subjected to phenotypic assimilation during the developmental process. The analysis of multiple phenotypes and multiple environmental factors is required to gain a more complete understanding of how the environment influences the phenotypic characteristics of individuals.

5.7 Conclusion

The main conclusion of this study is that there is an effect of temperature and water flow on vertebral number and body shape in *A. mexicanus*. The shape variation differs between temperature and water flow. This effect is multidimensional; morphological change due to alterations in the environment can potentially, affect other traits. The induced morphological changes in individuals were the result of differences in temperature during their development, and later the influence of water flow regimes. These simple changes could lead to more complex responses in nature. When placing these findings in a broader context, the rise of temperatures due to global climate change can become even more significant. Experiments like this one may help to understand how species may adapt to future environmental change. The findings of this study, could potentially apply to other characids that may be experiencing rapid changes in their habitat as well. Individually, the results of temperature and flow are consistent with other studies, and they seem to produce an additive effect on body shape variation. Water flow specifically seems to affect shape somewhat independently of temperature. Also, studies on the effects of temperature on embryos could provide more information on how developmental pathways are altered by changes in the environment. Future studies should try to link the observed responses to
other functionally important traits. Swimming performance is often used as factor to evaluate the fitness of individuals and other studies had suggested that variation in the number of vertebrae and in body shape may influence how individual fish swim (Batty & Blaxter, 1992; Swain, 1992a; Brainerd & Patek, 1998). Conducting analysis that link the phenotypic variation observed to a behavioral trait would provide a more complete picture of how plasticity impacts individual performance.


Zebradish, 8(4), 155-165.


Hubbs, C. L. (1922). Variations in the number of vertebrae and other meristic characters of fishes correlated with the temperature of water during development. The American Naturalist, 56(645) 360-372.

Hubbs, C. L. (1926). The structural consequences of modifications of the developmental rate in fishes, considered in reference to certain problems of evolution. The American Naturalist, 60(666) 57-81.


Kimura, T., Shinya, M., & Naruse, K. (2012). Genetic analysis of vertebral regionalization and number in medaka (Oryzias latipes) inbred lines. G3: Genes|Genomes|Genetics, 2(11), 1317-1323. doi: 10.1534/g3.112.003236


Kwain, W.-h. (1975). Embryonic development, early growth, and meristic variation in rainbow trout (Salmo gairdneri) exposed to combinations of light intensity and temperature. Journal of the Fisheries Research Board of Canada, 32(3), 397-402. doi: 10.1139/f75-046


7 TABLES AND FIGURES

Table 1. Frequencies of vertebral phenotypes grouped by treatments. T. = Temperature treatments. F = Water flow treatments. 0 = No flow, 1 = Water flow. Total column, total frequency of individuals per treatments

<table>
<thead>
<tr>
<th></th>
<th>Precaudal vertebras</th>
<th>Caudal Vertebrae</th>
<th>Total Vertebrae</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>12 13 14 14 15 16 17 18 19 27 28 29 30 31 32</td>
<td>20 21 22 23 24 25 26 27 28 29 30 31 32</td>
<td>Total</td>
</tr>
<tr>
<td>T</td>
<td>F.</td>
<td>12</td>
<td>13</td>
</tr>
<tr>
<td>20</td>
<td>0</td>
<td>1</td>
<td>30</td>
</tr>
<tr>
<td>20</td>
<td>1</td>
<td>1</td>
<td>27</td>
</tr>
<tr>
<td>23</td>
<td>0</td>
<td>2</td>
<td>33</td>
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<tr>
<td>23</td>
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<td>5</td>
<td>31</td>
</tr>
<tr>
<td>25</td>
<td>0</td>
<td>3</td>
<td>32</td>
</tr>
<tr>
<td>25</td>
<td>1</td>
<td>2</td>
<td>34</td>
</tr>
<tr>
<td>28</td>
<td>0</td>
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<td>28</td>
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<td>31</td>
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<tr>
<td>Total</td>
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<td>251</td>
<td>42</td>
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Table 2. Size correction analysis. A) Two-way factorial ANOVA conducted to examine whether centroid size differs significantly among temperature or water flow treatments. B) Linear regression results of shape variation on size.

A) ANOVA Summary Centroid size

<table>
<thead>
<tr>
<th>Source</th>
<th>SS</th>
<th>df</th>
<th>MS</th>
<th>F</th>
<th>P</th>
</tr>
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<tr>
<td>Flow</td>
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<td>1</td>
<td>3494</td>
<td>0.26</td>
<td>0.615</td>
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<tr>
<td>Temp.</td>
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<td>65170</td>
<td>4.78</td>
<td>0.01</td>
</tr>
<tr>
<td>F x T</td>
<td>99779</td>
<td>3</td>
<td>33260</td>
<td>2.44</td>
<td>0.089</td>
</tr>
<tr>
<td>Error</td>
<td>3E+05</td>
<td>24</td>
<td>13642</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Total</td>
<td>6E+05</td>
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<td></td>
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</table>

B) Regression results

<table>
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<tr>
<th>Sums of squares</th>
<th></th>
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<tbody>
<tr>
<td>Total SS:</td>
<td>0.004361</td>
</tr>
<tr>
<td>Predicted SS:</td>
<td>0.000295</td>
</tr>
<tr>
<td>Residual SS:</td>
<td>0.004066</td>
</tr>
<tr>
<td>% predicted:</td>
<td>6.76%</td>
</tr>
<tr>
<td>P</td>
<td>0.0368</td>
</tr>
</tbody>
</table>
Table 3. A. Results of Chi square test of independence between the presence and absence of vertebral fusions and temperature treatments. Chi square test of independence between the presence and absence of vertebral fusions (Fused Vert.) and water flow treatments.

A)

<table>
<thead>
<tr>
<th>Temperature</th>
<th>20</th>
<th>23</th>
<th>25</th>
<th>28</th>
<th>Total</th>
</tr>
</thead>
<tbody>
<tr>
<td>No Fused Vert.</td>
<td>45</td>
<td>67</td>
<td>60</td>
<td>41</td>
<td>213</td>
</tr>
<tr>
<td>Fused Vert.</td>
<td>32</td>
<td>13</td>
<td>20</td>
<td>35</td>
<td>100</td>
</tr>
<tr>
<td>Total</td>
<td>77</td>
<td>80</td>
<td>80</td>
<td>76</td>
<td>313</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Chi-Square</th>
<th>df</th>
<th>p</th>
</tr>
</thead>
<tbody>
<tr>
<td>21.07</td>
<td>3</td>
<td>&lt;0.001</td>
</tr>
</tbody>
</table>

B)

<table>
<thead>
<tr>
<th>No flow</th>
<th>Flow</th>
<th>Total</th>
</tr>
</thead>
<tbody>
<tr>
<td>No Fused Vert.</td>
<td>106</td>
<td>107</td>
</tr>
<tr>
<td>Fused Vert.</td>
<td>50</td>
<td>50</td>
</tr>
<tr>
<td>Total</td>
<td>156</td>
<td>157</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Chi-square</th>
<th>df</th>
<th>p</th>
</tr>
</thead>
<tbody>
<tr>
<td>0.01</td>
<td>1</td>
<td>0.923</td>
</tr>
</tbody>
</table>
Table 4. Influence of centroid size (Cnt. Size), temperature (20°C, 23°C, 25°C, 28°C), flow, temperature x flow, total vertebral number and vertebral ratio (Pre/Cau) on body shape. Significant variables are in bold. Wilk’s partial $\eta^2 = \frac{1-\lambda^{1/s}}{s}$, where $s = \min(p, df_{effect})$, $p$ = number of dependent variables in a factor, and $df_{effect}$ = degrees of freedom of the factor of interest.

<table>
<thead>
<tr>
<th></th>
<th>Wilk's $\lambda$</th>
<th>$F_s$</th>
<th>$df_1$</th>
<th>$df_2$</th>
<th>$P$</th>
<th>Wilk's partial $\eta^2$</th>
<th>Rank</th>
</tr>
</thead>
<tbody>
<tr>
<td>Cnt. size</td>
<td>0.65</td>
<td>5.52</td>
<td>26</td>
<td>277</td>
<td>&lt;0.001</td>
<td>0.35</td>
<td>1</td>
</tr>
<tr>
<td>Temp</td>
<td>0.44</td>
<td>3.28</td>
<td>78</td>
<td>829</td>
<td>&lt;0.001</td>
<td>0.24</td>
<td>2</td>
</tr>
<tr>
<td>Flow</td>
<td>0.78</td>
<td>2.98</td>
<td>26</td>
<td>277</td>
<td>&lt;0.001</td>
<td>0.22</td>
<td>3</td>
</tr>
<tr>
<td>T x F</td>
<td>0.7</td>
<td>1.32</td>
<td>78</td>
<td>829</td>
<td>0.0369</td>
<td>0.11</td>
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</tr>
<tr>
<td>Total vert.</td>
<td>0.85</td>
<td>1.83</td>
<td>26</td>
<td>277</td>
<td><strong>0.009</strong></td>
<td>0.15</td>
<td>5</td>
</tr>
<tr>
<td>Vert Ratio</td>
<td>0.83</td>
<td>2.03</td>
<td>26</td>
<td>277</td>
<td><strong>0.002</strong></td>
<td>0.17</td>
<td>4</td>
</tr>
</tbody>
</table>
Table 5. Pairwise values of Mahalanobis distance between treatment groups. Top: Mahalanobis distances. Bottom: p-values from permutation tests (10,000 permutation rounds). Significant values are in bold.

<table>
<thead>
<tr>
<th>M dist.</th>
<th>20-NF</th>
<th>20-F</th>
<th>23-NF</th>
<th>23-F</th>
<th>25-NF</th>
<th>25-F</th>
<th>28-NF</th>
<th>28-F</th>
</tr>
</thead>
<tbody>
<tr>
<td>20-F</td>
<td>1.6989</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>23-NF</td>
<td>2.2849</td>
<td>2.2989</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>23-F</td>
<td>2.4312</td>
<td>2.3092</td>
<td>1.6986</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>25-NF</td>
<td>2.0310</td>
<td>1.9244</td>
<td>1.1792</td>
<td>1.3777</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>25-F</td>
<td>2.3477</td>
<td>1.8819</td>
<td>1.9668</td>
<td>1.6562</td>
<td>1.3011</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>28-NF</td>
<td>2.3800</td>
<td>2.2796</td>
<td>2.0639</td>
<td>1.9792</td>
<td>1.5641</td>
<td>1.6397</td>
<td></td>
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</tr>
<tr>
<td>28-F</td>
<td>2.6764</td>
<td>2.1633</td>
<td>2.1446</td>
<td>1.9267</td>
<td>1.5305</td>
<td>1.3652</td>
<td>1.6032</td>
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</tr>
<tr>
<td>p-values</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
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<td></td>
</tr>
<tr>
<td>20-NF</td>
<td></td>
<td></td>
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<td></td>
</tr>
<tr>
<td>20-F</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>23-NF</td>
<td>&lt;.0001</td>
<td>&lt;.0001</td>
<td>0.0001</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>23-F</td>
<td>&lt;.0001</td>
<td>&lt;.0001</td>
<td>0.0001</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>25-NF</td>
<td>&lt;.0001</td>
<td>&lt;.0001</td>
<td>0.2694</td>
<td>0.0173</td>
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</tr>
<tr>
<td>25-F</td>
<td>&lt;.0001</td>
<td>&lt;.0001</td>
<td>&lt;.0001</td>
<td>&lt;.0001</td>
<td>0.0003</td>
<td>0.0529</td>
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<td></td>
</tr>
<tr>
<td>28-NF</td>
<td>&lt;.0001</td>
<td>&lt;.0001</td>
<td>&lt;.0001</td>
<td>&lt;.0001</td>
<td>&lt;.0001</td>
<td>0.0031</td>
<td>0.0018</td>
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</tr>
<tr>
<td>28-F</td>
<td>&lt;.0001</td>
<td>&lt;.0001</td>
<td>&lt;.0001</td>
<td>&lt;.0001</td>
<td>&lt;.0001</td>
<td>0.0035</td>
<td>0.0760</td>
<td>0.0119</td>
</tr>
</tbody>
</table>
Table 6. Discriminant function analysis results between water flow and no flow treatments. Mahalanobis distance between treatment means: 1.0269. Procrustes distance: 0.0070. Left: discriminant function allocations. Right: Allocations after cross-validation. The allocations are significant after 1000 permutation runs (p<.0001).

<table>
<thead>
<tr>
<th>True Group</th>
<th>Water flow</th>
<th>No flow</th>
<th>Total</th>
</tr>
</thead>
<tbody>
<tr>
<td>Water flow</td>
<td>71.97</td>
<td>28.03</td>
<td>100</td>
</tr>
<tr>
<td>No Flow</td>
<td>28.85</td>
<td>71.15</td>
<td>100</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>True Group</th>
<th>Water flow</th>
<th>No flow</th>
<th>Total</th>
</tr>
</thead>
<tbody>
<tr>
<td>Water flow</td>
<td>109</td>
<td>48</td>
<td>157</td>
</tr>
<tr>
<td>No Flow</td>
<td>55</td>
<td>101</td>
<td>156</td>
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</table>
Table 7. Mahalanobis distances in body shape among specimens differing in vertebral number within temperature treatment. The P-value from permutation test (10000 permutation rounds) among groups. A. 20°C; B. 23°C, C. 25°C, D. 28°C

<table>
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<tr>
<th>A</th>
<th>20°C</th>
<th>29</th>
<th>30</th>
<th>31</th>
</tr>
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<tbody>
<tr>
<td>30</td>
<td>3.3068</td>
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<td></td>
<td></td>
</tr>
<tr>
<td>31</td>
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<td>32</td>
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<td>29 30 31</td>
<td></td>
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<td></td>
</tr>
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<tr>
<td>31</td>
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<td>32</td>
<td>0.0629 0.0347 0.0087</td>
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</table>

<table>
<thead>
<tr>
<th>B</th>
<th>23°C</th>
<th>28</th>
<th>29</th>
<th>30</th>
<th>31</th>
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</thead>
<tbody>
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<td>29</td>
<td>11.4414</td>
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</tr>
<tr>
<td>30</td>
<td>8.8897 7.4544</td>
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<td>9.1163 7.7541 1.6352</td>
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Figure 1. X-ray of *Astyanax mexicanus* indicating method for counting vertebrae and major anatomical structures. Vertebrae are labelled with individual landmarks 1-30. W, Weberian apparatus (not included in counts); Pre, Precaudal, includes the vertebrae forming the rib cage (1-13); Cau, caudal vertebrae include vertebrae with hemal spines (14-31). Vertebrae 14 and 15 are transitional vertebrae and were included in the counts of caudal vertebrae because they have small hemal spines and lack ribs. U, urostyle (not included in vertebral counts). The star indicates fused vertebrae that were counted as vertebrae 19 and 20. Notice the extra hemal spines and the junction formed in the middle of the vertebral body.
Figure 3. Total percentage of individuals with fused vertebrae per temperature treatments. 20ºC: 42% of 77 specimens, 23ºC: 16% of 80 specimens, 25ºC: 25% of 80 specimens, 28ºC: 46% of 76 specimens. B. Plot of fused vertebrae by temperature treatments. Each point represents a tank. Tanks with the same frequencies are plotted next to each other for clarity. Each treatment has eight tanks.
Mean vertebral counts
30.2 30.3 30.4 30.5 30.6 30.7 30.8 30.9

Vert. Ratio (#Precaudal/#Caudal)
0.72 0.73 0.74 0.75 0.76 0.77

Figure 4. A. Mean total number of vertebrae by temperature treatment. B. Vertebral ratio (#Precaudal/#Caudal). Error bars: Standard error of the mean. NF= No flow; F= Flow treatments.
Figure 5. Plot of the mean number of precaudal vertebrae vs. the mean number caudal vertebrae coded by treatment. Error bars: Standard error.
Figure 6. Predicted body shapes for specimens differing in centroid size. A. Predicted body shape for small individuals. B. Predicted body shape for large individuals. Figures are exaggerated 3 times.
Figure 7. Top. PCA of body shape variation of all specimens included in the study. The PCA was conducted on the individual specimen data but points are tank means coded by treatments to facilitate interpretation. The percentage represent the amount of variance explained by each PC. Bottom, predicted shapes at the extremes of PC1 and PC2. A. PC1 negative extreme. B. PC1 positive extreme. C. PC2 negative extreme. D. PC2 positive extreme. The black frame represents the consensus shape of the PCs, the gray frame represents the consensus configuration of all specimens for comparison.
Figure 8. Canonical variate analysis (CVA) of body shape variation conducted using individual size-corrected specimen data, taking into account membership in both the temperature and water flow treatments. Top: CVA plot with tank means as data points coded by treatment. Open symbols represent no water flow treatments. Closed symbols, represent water flow treatments. Temperature is color coded: Blue: 20°C, Green: 23°C, Pink: 25°C, Red: 28°C. Percentages indicate the variation explained by each CV. Bottom: Predicted shapes for the extremes of CV1 and CV2. A. Predicted shape of CV1 towards the negative end. B. Predicted shape of CV1 on the positive end. C. Predicted shape of CV2 on the negative end. D. Predicted shape of CV2 at the positive end. Shapes are exaggerated by 10 times.
Figure 9. Top. Plot of the CVA conducted to analyze body shape variation in specimens based on the water flow treatments using size corrected data. Open symbols indicate the no water flow treatments and closed symbols indicate water flow treatments. Colors represent temperature treatments. Blue: 20°C, Green: 23°C, Pink: 25°C, Red: 28°C. A single CV axis resulted from this analysis because only two groups were designated (flow and no flow). Mahalanobis distance between group means: 1.027, p = <0.001; Procrustes distance: 0.0055, p = 0.002). Bottom. Predicted consensus shapes of individuals at the extremes of the CV axis. A. No water flow treatment. B. Water flow treatment. Shapes are exaggerated by 10 times.
Figure 10. Predicted body shapes of specimens based on the regression of body shape on vertebral number and vertebral ratio. A. Predicted shape for individuals with a low total number of vertebrae. B. Predicted shape for individuals with a high number of total vertebrae. C. Predicted shape of individuals with a higher proportion of caudal vertebrae. D. Predicted shape of individuals with higher proportion of precaudal vertebrae. Shapes exaggerated by 10 times.
Figure 11. Series of CVAs of body shape variation with groups designated by total vertebral number. A separate CVA was conducted for each temperature treatment. A. 20°C. B. 23°C. C. 25°C. D. 28°C. Colors represent total vertebral number. 27: Yellow, 28: Light green, 29: Dark green, 30: Pink, 31: Blue and 32: Red
## APPENDIX

**Sup. Table 1.** Mean vertebral counts per tank. Treatments: 0 = No flow, 1 = Flow. 20-28 refer to the temperature treatments. #Precaudal is the number of precaudal vertebrae. #Caudal is the number of caudal vertebrae. Total is the total number of vertebrae. Ratio Pre/Cau is the ratio of precaudal to caudal vertebrae.

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**Sup. Table 2.** Percentage of difference between treatment groups, using the highest Mahalanobis distance as 100% indicated in bold, and the others are scaled by that reference.

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Sup. Table 3. CVA per temperature results. A) Mahalanobis distance between temperatures. B) Percentage difference between treatment groups using the highest Mahalanobis distance as 100% indicated in bold, and the others are scaled by that reference. All distances were significant (p<0.0001)

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Sup. Table 4. Percentage of difference temperature groups, using the highest Mahalanobis distance as 100% indicated in bold, and the others are scaled by that reference. A. 20°C. B. 23°C. C. 25°C D. 28°C.

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Sup. Fig. 1. Percentage of differentiation based on the Mahalanobis distances of the CVA per treatments.
Sup. Fig. 2. Canonical variate analysis by temperature treatments using the data corrected for size effects. The plot represents the mean of the individual’s data. Open symbols represents no water flow treatments. Closed symbols, represents water flow treatments. Temperature is color coded: Blue: 20ºC; Green: 23ºC; Pink: 25ºC; Red: 28ºC. Percentages indicates the variation explained by each CV.
Sup. Fig. 3. Percentage of differentiation based on the Mahalanobis distances of the CVA per temperatures.
Sup. Fig. 4. Percentage of differentiation based on the Mahalanobis distances of the CVA of total vertebral counts per temperatures.
Sup. Fig. 5. Predicted consensus shapes of total vertebral number at 20°C. A. Consensus shape of CV1 towards the negative end. B. Predicted shape of CV1 on the positive end. C. Consensus shape of CV2 on the negative end. D. Consensus shape of Cv2 in the positive end.
Sup. Fig. 6. Predicted consensus shapes of total vertebral number at 23°C. A. Consensus shape of CV1 towards the negative end. B. Predicted shape of CV1 on the positive end. C. Consensus shape of CV2 on the negative end. D. Consensus shape of Cv2 in the positive end.
Sup. Fig. 7. Predicted consensus shapes of total vertebral number at 25°C. A. Consensus shape of CV1 towards the negative end. B. Predicted shape of CV1 on the positive end. C. Consensus shape of CV2 on the negative end. D. Consensus shape of Cv2 in the positive end.
Sup. Fig. 8. Predicted consensus shapes of total vertebral number at 28°C. A. Consensus shape of CV1 towards the negative end. B. Predicted shape of CV1 on the positive end. C. Consensus shape of CV2 on the negative end. D. Consensus shape of Cv2 in the positive end.