Summer 8-21-2022

Temperature Effects on the Development of the Axial Skeleton and Body Shape in Astyanax mexicanus (Teleostei: Characidae)

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Temperature Effects on the Development of the Axial Skeleton and Body Shape in *Astyanax mexicanus* (Teleostei: Characidae)

A Thesis

Presented in

Partial Fulfillment of the

Requirements for the Degree of

Master of Science

By

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August, 2022

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Chicago, Illinois

Contents:

Acknowledgments:

To begin, I would like to show a deep appreciation for my advisor Dr. Windsor Aguirre; without him, this project would have never come to fruition. His passion for the field of evolution is extremely contagious and caused my interest to increase even more. His thoughtfulness, expertise, and intelligence helped guide me through this project that I am incredibly thankful to have as a mentor in a graduate program. He helped me grow as a student, academic, and independent throughout these many years, and I am again genuinely thankful for all I have learned from him.

I would like to show gratitude to my thesis committee members Dr. Elizabeth LeClair, Dr. Jalene LaMontagne, and Dr. Kenshu Shimada. Not only have they guided me in my project and thesis, but they also were instructors of courses I took that helped advanced my knowledge in science that aided my work. I would specifically like to thank Dr. LeClair for her wisdom in developmental biology that I was very new to when I entered this graduate program, and to Dr. LaMontagne for her expertise in advanced statistics and operating the program R. Without them I would still be analyzing data. I would like to thank Dr. Shimada for stepping up to being a committee member with the departure of Dr. LeClair, without him I would not be able to defend my thesis.

My appreciation is also extended to the biological sciences faculty who educated, supported, and inspired me throughout this journey by loaning me equipment, introducing me to graduate school life, and increasing my knowledge in many areas of biology.

Finally, I would like to thank my family, thesis cohort, and Aguirre Lab members for their unconditional support through my masters and encouraging me when the going got tough.

Abstract:

Humans are causing large-scale changes in environmental conditions across the planet including in temperature. Changes in the environmental conditions can lead to phenotypic changes in ectotherms that affect adaptively important traits like body shape and the axial skeleton. Previous studies have shown that temperature changes during development significantly affects body shape and vertebral number in the Mexican tetra *Astyanax mexicanus*. How these changes arise early in development is not clear. In this study, I examine how changes in developmental temperature affect body shape in larval and juvenile fish, the order of ossification of elements of the axial skeleton, the size of set bone ossification, and the variation in size of set ossification of different skeletal structures. Fertilized eggs from laboratory reared *A. mexicanus* were maintained at 20°C, 24°C, and 28°C, then collected, preserved, imaged, cleared, and stained with alizarin red to examine bone ossification.

Temperature significantly influenced the body shape of larval *A. mexicanus*, resulting in fish in the 28°C water treatment having deeper bodies compared to fish reared at 20°C. There was not a significant relationship between temperature and the order of bone ossification, indicating that bone developmental order is constrained across the range of developmental temperatures examined. However, temperature significantly affected the size of ossification of certain skeletal structures with the bones of fish reared at 20°C ossifying at larger sizes than fish reared at 24°C and 28°C. There was also a relationship between temperature and the variation in set size of bone ossification among crosses. Fish developing at 20°C tended to exhibit the most variability in the size of set ossification among crosses.

This study examens whether different rearing temperatures can affect the early stages of larval development thus resulting in modified phenotypes of adult *A. mexicanus*. My research

also provides a baseline for future studies examining phenotypic plasticity in body shape and skeletal ossification in fish colonizing new habitats or fish in rapidly changing environments.

Chapter 1:

Introduction:

Evolution in its simplest form is the change in the genetic properties of a population over time. Phenotypic plasticity is a non-evolutionary mechanism of phenotypic change present in some organisms in which a single genotype can produce different phenotypes in different environments (West-Eberhard, 2003). Although phenotypic plasticity has traditionally been thought of as an alternative mechanism for phenotypic change relative to evolution, it is now understood that plasticity can facilitate adaptive evolution in some cases by allowing populations to survive sudden changes in environmental conditions and by influencing the direction of evolution (West-Eberhard, 2003; Wund et al., 2008).

Ectotherms like fish are frequent targets of studies of phenotypic plasticity because their body temperature varies with the environmental temperature, and because temperature is one of the most important factors causing changes in the phenotypic properties of organisms (Bird and Mabee, 2003). Temperature changes typically results in the slowing down or accelerating of development in ectotherms, which can have significant effects on their phenotypic properties (Bird and Mabee, 2003; Barriga et al., 2013; Reyes and Aguirre, 2019). Responses to temperature changes can also be more complex, resulting in heterogeneous rates of development in different traits at different temperatures, and phenotypic differences among individuals when compared at the same developmental stage of body size (Boughton et al., 1991). How these mismatches in developmental rates arise is not well understood, nor are their functional implications. My thesis aims to address some gaps in knowledge in the morphological implications of temperature changes in fishes.

These are important areas of biology because they are critical for understanding how biological diversity originates. This review provides background information on topics central to the research conducted in Chapter 2. The review starts addressing more general topics like evolution and development and then narrows down to more specific topics related to how phenotypic plasticity affects fish development, canalization, vertebral development, Jordan's Rule, and why characids were used for exploring this line of research.

My experimental research is described in Chapter 2 and involves two major components. First, I examine body shape variation through the early development of specimens of *Astyanax mexicanus* reared at different temperatures using geometric morphometrics to determine how temperature affects body shape development in this species. I am especially interested in whether temperature differences result in significant differences in the body shape of young specimens once differences in developmental rate and body size are accounted for. This will help to determine when previously documented temperature induced differences in body shape in this species arise during development (Reyes and Aguirre, 2019). Second, I examine how developmental temperature affects the order in which bones of the axial skeleton develop and the size at which they ossify.

Literature review:

Phenotypic Plasticity and Development:

Phenotypic plasticity is when an individual responds to changes in its environment by developing a different phenotype. Many environmental factors can contribute to plasticity of a species like food availably, absence or presence of predators, competition, and temperature, all of which affect the phenotypes of many fish species (Georgakopoulou et al., 2007). Temperature is a particularly important factor affecting fishes and other ectotherms (Jordan, 1892; Georgakopoulou et al., 2007; McDowall, 2008; Barriga et al., 2013; Ramler et al., 2014; Ackerly and Ward, 2015; Reyes and Aguirre, 2019). For example, depending on the temperature of development, fish will respond by having different vertebral column phenotypes (McDowall, 2008; Barriga et al., 2013; Reyes and Aguirre, 2019). Plasticity not only changes an individual's phenotype without affecting the genotype but can also increase the phenotypic variability observed within species subjected to heterogeneous environmental conditions throughout their range. These changes in phenotype can also help in niche construction and niche partitioning by the individuals with altered phenotypes (Gilbert et al. 2015). Lastly, these initially plasticity-induced phenotypic changes can evolve in some cases and become assimilated into the genome as inherited traits (Gilbert et al. 2015).

Assimilation of a trait into the genome of the organism gained recognition with Waddington's (1953) heat shock study of *Drosophila melanogaster*. After receiving a heat shock of 40°C for 4 hours, some *D. melanogaster* pupae developed crossveinless wings (Waddington, 1953). Upon further breeding for several generations, *Drosophila* offspring started developing crossveinless wings without the heat shock, meaning the trait for crossveinless wings assimilated into the genome of *D. melanogaster* (Waddington, 1953). Looking at how a certain trait has been influenced by its environment, and how that potentially can influence the evolutionary trajectory

of lineages may be important for understanding the evolution of biological diversity (Mabee et al., 2000). With all the potential effects that phenotypic plasticity has on organisms, there are many questions that remain unanswered. For example, how do these differences arise, that is, what exactly are the developmental changes causing the differences seen in adults?

Canalization:

The belief that an organism's phenotype is universally plastic and responsive to its surrounding environment comes with opposition, specifically with the concept of canalization. Canalization is the concept that species are developmentally robust (Siegal and Bergman, 2002). This robustness is said to be evolved from a history of natural selection that has been selecting for the best phenotype in a given environment for many generations. What is observed is that most species have substantial genetic variation, and experience significant variation in environmental conditions as they develop, yet phenotypic variation from individual to individual is often relatively low (Siegal and Bergman, 2002). This low phenotypic variation is attributed to the evolution of physiological mechanisms to direct development through a restricted range of possible phenotypes. Developmental robustness is seen in almost all species. However, ectotherms, like fish, can experience more extreme variation in environmental conditions during development than endotherms, like mammals and birds, because their internal body temperature varies with the environmental temperature. Studies of fishes indicate that adaptively important traits like body form and vertebral number are highly responsive to environmental variation (McDowall, 2008; Barriga et al., 2013; Aguirre et al., 2014; Ramler et al., 2014; Reyes and Aguirre, 2019). Similarly, organisms like nematodes and *Daphnia* are both highly responsive to their environment in morphology (Scheiner and Berrigan, 1998; Nijhout, 2015). It is unclear

whether adaptive plasticity related to temperature variation is greater in ectotherms like fishes than in endotherms because of their likely historical exposure to greater variation in temperature during development.

Vertebrae and Jordan's Rule:

An important form of variation seen in animals is the variation in the number and identity of vertebral segments (Yamahira and Nishida, 2009). Species vary immensely in the number of vertebrae and the proportion of each type of vertebrae, from eels having 115 to 160 vertebrae to trunkfish having only 14 (Jordan, 1892). Fishes are particularly variable in body form and segment number. This diversity of the axial patterning, and subsequently the morphological diversity, may contribute to why there are more fishes than all other vertebrate taxa combined (Yamahira and Nishida, 2009; Ackerly and Ward, 2015). Although axial diversity is primarily due to genetic differences among fish lineages, the axial skeleton of fishes is also influenced by temperature. For example, it is known that geography, specifically latitude, affects vertebral number. This geographic influence is commonly known as 'Jordan's Rule' (Yamahira and Nishida, 2009). Jordan's rule simply stated is: 'in certain groups of fishes from the northern or cold-water representatives have a larger number of vertebrae than those members which are found in tropical regions' (McDowall, 2008). Jordan (1892) noted that this trend was seen in blenny like fishes, where he noted that the tropical genera have 28 to 49 vertebrae and the arctic genera having 75 to 100. Jordan's Rule has also been applied to another geographic change: change in altitude (Barriga et al., 2013). As with latitude, as altitude increases, vertebral number may increase in some lineages. When looking at Jordan's Rule one can substitute 'higher latitude' and 'altitude' with 'colder water temperature', leading to the conclusion that as water

temperature decreases vertebral number increases. Jordan's Rule appears to be relatively common and has been reported in many fishes (McDowall, 2008; Barriga et al., 2013; Morris et al., 2018).

The Importance of the Characidae and *Astyanax mexicanus*:

The organism used in this study is the fish species *Astyanax mexicanus*, the Mexican tetra, which is in the family Characidae. The Characidae is the most diverse family of fishes in freshwaters of the Americas, with over 2000 species across the globe, and 88% of those being in Central and South America (Mattox et al., 2014). Characids have been able to adapt to many habitats and are one of the most prevalent families in the neotropics (Jeffery, 2008; Mattox et al., 2014). They also occur at different latitudes and along elevational gradients in mountain streams and thus experience highly varied environmental conditions.

Astyanax mexicanus is becoming a model species in evolutionary development for several reasons. For one, cave forms that are extremely divergent phenotypically from the surface forms have evolved independently in Mexico several times (Coghill et al., 2014). Individuals of this species are also small, easily raised in a laboratory with a simple diet, and have a short generation time of 4-6 months (Jeffery, 2008). They can produce hundreds of individuals in a single clutch. Their developmental pattern is closely related to that of other teleosts, which are the most diverse of vertebrates and can easily be observed and studied because of their large, transparent embryos (like zebrafish) (Jeffery, 2001; Woltering et al., 2018). *Astyanax mexicanus* are also related phylogenetically to zebrafish, both being members of the Superorder Ostariophysi. This relationship to a major model organism for development, allows for similar experimental procedures to be conducted on them (Jeffery, 2001).

Chapter 2

Introduction:

Phenotypic plasticity, the organism's ability to acclimate to its environment without an alteration in its genetic makeup, is an important mode of responding to environmental challenges (West-Eberhard, 2003). Although adaptive evolution and phenotypic plasticity have traditionally been thought of as alternative mechanisms for phenotypic change, it is now understood that plasticity can facilitate adaptive evolution by allowing populations to survive sudden changes in environmental conditions and by potentially influencing the direction of evolution (West-Eberhard, 2003; Wund et al., 2008). Understanding phenotypic plasticity also has practical implications. For example, as global temperatures rise (IPCC, 2014), understanding how changes in temperature affect developmental patterns and the phenotypes of adult organisms may provide insight into why some species successfully adjust while others do not (West-Eberhard, 2003; Wund et al., 2008**)**.

Most fishes are particularly responsive to their environment and temperature variation because they are ectotherms. Many fishes including zebrafish (*Danio)*, stickleback (*Gasterosteidae)*, and the Mexican tetra (*Astyanax mexicanus)*, respond to temperature variation by either slowing down or accelerating their development (Bird and Mabee, 2003; Barriga et al., 2013; Reyes and Aguirre, 2019). However, there is still much to be learned about the morphological implications of these changes on the development of fishes. For example, Reyes and Aguirre (2019) found that vertebral number diverged significantly among specimens of the Mexican tetra reared at different temperatures, with the effect being most pronounced in the precaudal vertebrae. Although it is clear that temperature variation can result in changes in the axial skeleton, it is not clear how these changes arise during development.

There is substantial diversity in the axial skeleton of fishes leading to great variation in body form, which is mostly due to genetic differences, but can also be caused by environmental variation during development (Yamahira and Nishida, 2009; Ackerly and Ward, 2015). Jordan's Rule is when the number of vertebrae increase as fish reach the poles, aka, the number of vertebrae increase as temperature decreases (Jordan, 1892; McDowall, 2008; Barriga et al., 2013). Jordan's Rule appears to be relatively common in fishes and has been reported in many species including stickleback, galaxiids, and characids, in both the Northern and Southern hemispheres (McDowall, 2008; Barriga et al., 2013; Morris et al., 2018; Reyes and Aguirre, 2019).

Reyes and Aguirre (2019) found that body shape and vertebral number varied in response to temperature variation. The number of precaudal vertebrae differed significantly among fish reared at different temperatures in *A. mexicanus,* but the number of caudal vertebrae and total number of vertebrae did not. However, the total number of vertebrae was only marginally non-significant (Reyes and Aguirre, 2019). Variation in vertebral number followed Jordan's rule with individuals reared at 20°C having the highest mean total number of vertebrae. Variation in the number of precaudal vertebrae followed the same trend, where the 20°C fish had the highest mean number of precaudal vertebrae and fish in the warmest treatment of 28°C had the lowest mean number of precaudal vertebrae (Reyes and Aguirre, 2019). For the number of caudal vertebrae, individuals in the 20°C treatment had the lowest number whereas 28°C had the highest number suggesting that precaudal and caudal vertebrae are negatively correlated across temperatures, and that colder temperatures are biased towards having greater proportion of precaudal to caudal vertebrae than individuals in warmer temperatures. This leads to the question

of whether temperature variation affects other phenotypic traits like the sequence of bone ossification.

The relationship between phenotypic plasticity and adaptive evolution can be complex. By quantifying development with tools like geometric morphometric image analysis (GMIA), the gap between developmental biology and evolution can be bridged (Mayer et al., 2014). GMIA is a method for biological shape analysis, which implements the use of landmarks (anatomically homologous landmarks) and semilandmarks (points marking the outline of an object) around the body to quantify the shape (Mayer et al., 2014). Semilandmarks are particularly important when quantifying shape in developing organisms, because at different points in development, individuals often lack well-marked anatomical structures upon which to set fixed landmarks (Mayer et al., 2014).

The objectives of this study are to understand how and when the different morphological changes seen in *Astyanax* arise during development, and how larval fish development is affected by temperature. Specifically for body shape, I examined whether body shape differs among larval *Astyanax* reared at different temperatures when accounting for differences in developmental rate and body size. For the axial skeleton, I sought to examine whether there are differences in the order at which bones of the axial skeleton develop and in the set size of ossification among temperatures. I also investigated whether there are elements of the axial skeleton that are more variable in the set size of ossification. Reyes and Aguirre (2019) found that body shape and the ratio of precaudal to caudal vertebrae were influenced by changes in developmental temperature. However, they did not document when in development these effects arise or if they affect the development of other parts of the axial skeleton development. My hypothesis is temperature variation will have a significant impact on the pattern and

developmental rate of the axial skeleton and body shape in the Mexican tetra. I predicted that temperature would affect body shape by making warmer treatments be deeper bodied in larval forms and the axial skeleton will have the more extreme temperature treatments will ossify bones at different sizes and in a different order. In this study, fish were bred and reared at three different temperatures (20°C, 24°C, and 28°C). Geometric morphometric image analysis was performed to examine how temperature affects body shape development and fry were then cleared and their skeleton stained to analyze the timing and sequence of bone ossification.

Materials and Methods:

Fish:

The experiment procedures were conducted under IACUC protocol 15-006. Four-year old individuals *Astyanax mexicanus* (surface morph) received from the Jeffery Laboratory at the University of Maryland and three-year old offspring of that colony were used in this study. This line of fish is a fourth and fifth generation laboratory strain that comes from wild-caught specimens from the Rio Grande in Texas (Reyes and Aguirre, 2019).

Breeding:

The fish were maintained under a 14-hour light and10-hour dark light cycle. Before breeding began, females were fed a high fat diet with Hikari Bio-Pure® freeze dried mealworms (65% crude protein) supplementing the normal TetraMin tropical fish flakes for two weeks. Males did not need to be prepared for breeding, so they were kept on the same diet. There was a total of ten breeding pairs established, only seven of which were used for this project: four pairs of Jeffery fish and three pairs of their offspring. To start breeding, the pairs were moved into breeding tanks the morning of the breeding with clean water at 21°C. To induce natural spawning, the temperature was raised to 24°C and monitored once the lights were turned off. Breeding was observed between 10pm and 2am. Upon successful breeding and fertilization, the eggs were gathered and distributed evenly into the different temperature treatments (Reyes and Aguirre, 2019).

Temperature Treatment:

The fertilized eggs were evenly distributed into the three treatments (20°C, 24°C, and 28°C +/- 1°C). To establish water temperatures, there were five petri dishes (9cm in diameter) for each cross in three different incubators set for each temperature. As eggs developed, I removed infertile and dead eggs. Once larvae lost their yolk sac, I began to feed them brine shrimp larvae. After reaching approximately 12 mm in length, I fed them ground-up flake food. These treatments continued until all fish were collected.

Tank Setup:

With temperatures established, 15 petri dishes were set up with 20 fish in each petri dish for the first half of fish development. The fish were housed in petri dishes, placed in incubators, until they reached approximately 12mm. At 12mm the fish were then moved to small plastic fish tanks (6L) heated by water baths in larger tanks (24°C, and 28°C) or a large cooling incubator (20°C).

Schedule of Fish Collection:

Temperature affects the rate of fish development (Bird and Mabee, 2003). Because the fish developed at different rates, each treatment group had a different collection schedule. From preliminary data collected, the schedule of collection was determined for each temperature treatment, with start dates staggered as not to miss bone ossification in any treatment. From the set start dates, collection varied slightly between crosses due to differences in total numbers and rate of survival between each cross. The 28°C treatment started collection 4 days postfertilization (DPF) at the earliest and ended 33 DPF at the latest. The 24°C collection began 6 DPF and ended

on 36 DPF. With the 20°C treatment, collections started 8 DPF and went consistently every day until 34 DPF for most treatments (cross 3 ended 49 DPF), and then continued every 2-3 days until 70 DPF when the last fish was collected.

Preservation:

Fish were preserved in a solution of 1 mL of 4% phosphate buffered paraformaldehyde and stored at 4°C until the clearing and staining process occurred.

Geometric Morphometric Analysis of Body Shape Variation:

Geometric morphometric image analysis (GMIA) was used to assess the effect of temperature on body shape over early development for each cross and each treatment group. Using GMIA allowed for qualitative shape variables among different fish samples to become transformed into quantitative variables for statistical analysis to examine if body shape differences occur between temperature treatments in fish smaller than 20mm in standard length.

Specimens were imaged using an Infinity1 microscope camera, Infinity Analyze software from Lumera, and a Discovery V12 Zeiss stereoscope. The images were digitized with TPSDig2 v2.31 (Rohlf, 2015). To analyze body shape variation, a mixture of 27 landmarks and sliding landmarks were placed along the body and eye. Landmarks were not placed in the ventral portion of the abdominal region because this area exhibited varying amounts of protrusion depending on the amount of food that had been recently consumed. Of the 27 landmarks placed, four were fixed landmarks (tip of snout, start of caudal fin, and two around the eye) and 23 were sliding landmarks to account for lack of homologous structures in larval fish (Fig. 1). The sliding landmarks were aligned using a Generalized Procrustes Analysis in TPSRelw v1.69 following

standard practices outlined for this procedure in the program manual (Rohlf, 2017) to account for variation in rotation, translation, and size (Zelditch et al., 2012). The aligned images allowed for analysis of body shape variation as a whole using all landmarks, rather than taking a few arbitrary linear measures to represent shape variation (Mayer et al., 2014).

Upon alignment of the sliding landmarks, a principal component analysis (PCA) was conducted in Morphoj (Klingenberg, 2011). PCA is an ordination method that takes large data sets with many variables and summarizes them in a lower dimensional space, where a dimension refers to a variable in this context. It does so by finding the major axes of variation in the data and rotating the space along these axes, allowing the visualization of body shape differences between groups in this lower dimensional space. The new PC axes are ordered in terms of the percentage of the original variation that they account for (Klingenberg, 2011; Zelditch et al., 2012; Reyes and Aguirre, 2019). Because body shape changes substantially over early development, PC1 was expected to be associated with body shape variation due to development. Body shape divergence for extreme values of each PC was used to determine the morphological correlates to each PC. Differences among temperature treatments in the PC scores were evaluated.

R version x64 3.5.1 and lme4 (Bates, Maechler and Bolker, 2012) were used to create a linear mixed effects model to examine the effects of temperature and the natural log of centroid size on shape PCAs, using cross as the random effect, with no interaction, in the model. Cross was included as a random effect to account for pseudoreplication of siblings residing in the same tanks and for genetic differences between families. Residual plots were used to evaluate homoscedasticity and normality. P-values were obtained from likelihood ratio tests of the full model with fixed effects against reduced models without either the fixed effect of centroid size,

temperature, or both. Models with different factors removed from them were run and compared to the models listed above to determine which model fit the data the best. The null models consisted of removing temperature as a factor, size as a factor, and a model with an interaction was used to determine if there was an interaction between size and temperature. Likelihood ratio tests on R were used to determine if there were statistical differences among the models which led me to choosing the model listed above.

Bone Staining and the Analysis of Developmental Timing:

The clearing and staining protocol for the larval fish was modified from Westerfield (2007) and Walker and Kimmel (2006). After the fish were fixed in paraformaldehyde all fish were rinsed with DI water and placed in 1% KOH and 3% hydrogen peroxide overnight to one day, depending on the stage of development, to depigment the fish. Then the larvae were placed in 30ml saturated sodium tetraborate in 70 ml of DI water overnight. The fish were then rinsed in DI water for ~30 minutes, then transferred to bone staining solution consisting of 1% KOH and 1mg/ml Alizarin Red for one day. After the staining stage, fish were rinsed for ~30 minutes with DI water then either transferred to a 1% trypsin in 2% sodium tetraborate solution for one day (fish 10mm standard length or longer) or moved directly to glycerol clearing solutions (fish <10mm standard length).

Fish that were transferred to the trypsin solution were placed in an incubator at ~30°C for optimal enzyme digestion for one day. All fish then went through a glycerol clearing sequence starting with 20% glycerol and 0.25% KOH, then 50% glycerol and 0.25% KOH, and finally a 50% glycerol and 0.1% KOH storage solution. Standard length was measured for all specimens,

and they were then imaged with the same equipment and programs used with geometric morphometric image analysis, and stored at 4°C.

For the bone development series, 18 bone structures were analyzed. They were: cleithrum, Weberian apparatus, first precaudal vertebra, last caudal vertebra, first and last rib, first and last haemal spine, first and last neural spine, first dorsal fin ray, first anal fin ray, first and last caudal fin ray, first and last hypural, dentary bone, and premaxilla (Fig. 2). A graph depicting the size of ossification of the skeletal structures listed above was created to visualize the differences in ossification among the temperature treatments. For this analysis, every specimen that exhibited ossification for at least one skeletal structure was included (Fig. 3). The order of the skeletal structures in the graph was determined by the average order of ossification across the temperature treatments.

To analyze ossification sequence, the standard length of ossification was taken for every fish in every cross for every bone structure. Structures were determined to be 'set' when every fish larger than the smallest also had the bone structure ossified. Any smaller fish with ossified structures were noted if there were fish larger in size without the structure ossified. These smaller fish are indicated by circles in the graph. The average set size of ossification for each temperature across all crosses was taken and graphed. To graph the results each structures' set size of ossification was graphed in rank order. Tied data were given the average score associated with the tied structures (Mabee et al., 2000). The average rank of each bone structure was taken between each temperature treatment to determine the order of bone ossification between treatments.

Each bone structure was then analyzed for differences in the order of ossification among temperature treatments. To compute the variation in the ossification rank of a bone, its largest

rank was subtracted from its lowest rank to obtain the difference between the temperature treatments (Mabee et al., 2000). A Spearman's correlation of ranks was used to measure the correlation of ranks of each bone structure ossification between temperatures. Spearman's correlation ranges from -1 to 1, with -1 indicating a perfect negative linear relationship, 1 indicating a perfect positive linear relationship, and 0 indicating no relationship. Kendall's coefficient of concordance was also used to examine how rank of ossification across traits compares between temperatures (Mabee et al., 2000). Kendall's coefficient of concordance (W) is calculated as $W= 12S/(m^2(n^3-n)-mT)$ where n is the number of objects (bone structures), m is the number of variables (temperature treatments), and T is a correction factor for tied ranks; measuring agreement among quantitative variables, in this case the order in which bones ossify throughout development (Legendre, 2010). Kendall's coefficient of concordance was calculated from the average ranks across crosses of the SL of the fish when each bone structure began ossifying. These rankings are then compared across each treatment to determine if there is concordance between the groups (Legendre, 2010). Kendall's coefficient of concordance varies between 0 and 1, with larger numbers indicating more agreement among ranks, and lower numbers indicating less agreement.

To analyze differences in set size of ossification, the coefficient of variation was calculated by taking the standard deviation divided by the mean and multiplying the number by 100, to analyze the variation of time of first appearance of each trait of each cross within temperature treatments to see if variation in standard length (SL) differed among groups. This measure of variation standardizes each variable's standard deviation by the mean, making it comparable across traits. A single factor ANOVA was used to determine if there were differences in the set body size of each cross, at which each skeletal structure ossified between

the temperature treatments. To account for multiple comparisons the Benjamini-Hochberg procedure was used as an alternative approach to control for false positive results. To determine the Benjamini-Hochberg critical value, each bone structure was put in order by smallest to largest P-value and ranked 1-18. The equation $(i/m)*Q$, where i is the rank, m is the total number of tests, and Q is the false discovery rate (I chose 0.20 to represent the 1/5 chance of a false positive for the 18 tests) gave the critical value to compare to the P-values. To determine significance, the largest P-value that is less than the calculated Benjamini-Hochberg value is found and all P-values smaller than that are also significant (Benjamini and Hochberg, 1995). Finally, a Tukey Post Hoc test was conducted to determine which groups differed from each.

Results:

Temperature Effects on Body Growth:

Temperature had a significant effect on developmental rate. When exposed to the 20°C treatment, the fish developed significantly slower than both 24°C and 28°C (Fig. 4a and 4b). At 29 DPF, the last day at which fish from all temperature treatments were collected in the crosses with large sample sizes (6, 8, 9, 10), 20°C fish had a centroid size of 2.81, 24°C fish had a centroid size of 3.06 (8.5% larger than 20C fish), and 28°C fish had a centroid size of 3.28 (15% larger than 20°C and 6.8% larger than 24°C). Because the effect of temperature on overall development rate is well known in fishes, the remaining analyses are described in relation to the natural log of centroid size to examine whether body shape and the axial skeleton differ between fish that developed at different temperatures when they are the same size.

Body Shape Variation:

The PCA indicated a relatively strong structuring of body shape variation among the specimens examined, with PC1 explaining 46.69% and PC2 explaining 17.13% of the variation in body shape. In total, the first two PCs accounted for 63.82% of the variation in body shape.

PC1:

Principal component 1 was strongly associated with body shape development (Fig. 5) as evidenced by the strong relationship between PC1 and age (r = 0.80; Fig. 6a and 6b) and size (r=0.78; Fig. 7a and 7b). Consequently, PC1 was considered an axis representing body shape variation associated with development. Fish went from having smaller heads with relatively large eyes, thin bodies, and undeveloped caudal regions in the earliest stages (Fig. 5b, negative end of PC1) to completely developed juvenile bodies with relatively smaller eyes, a larger head and jaw, a larger caudal region, and deeper bodies (Fig. 5a, positive end of PC1). Specimens from all the temperature treatments had generally similar body shapes on both the positive and negative ends, with slight deviations on the positive end of PC1.

The mixed effects model showed that temperature did not have a significant effect on PC1 when the log of centroid size and cross were considered (x^2=5.78, DF=2, P-value=0.055) (Table 1). This was also true when the crosses with small sample sizes (crosses 1, 3, and 5) were excluded (x^2=1.43, DF=2, P-value=0.490) (Table 2). Per unit size, 24°C (y-intercept=-0.266) fish had slightly larger scores for PC1, followed by the 28°C treatment fish (y-intercept=-0.270) and finally the 20°C fish (y-intercept=-0.273) (Fig. 7a). Plotting PC1 against Ln centroid size showed that the 20°C fish had conspicuously lower PC scores per unit size than the 24°C and 28°C fish, suggesting that their bodies were slightly less developed at the same size (Fig. 7a). This was not

the case when only the crosses with large sample sizes (crosses 6, 8, 9, and 10) were included (Fig. 7b). Per unit size fish were relatively the same for PC1 (20°C y-intercept=-0.267; 24°C y-intercept=-0.263; 28°C y-intercept=-0.269).

PC2:

There was substantial variation among individuals of similar length in body depth, which was associated with principal component 2. Fish on the positive end of PC2 had deeper bodies (Fig. 9b) compared to the negative end of PC2, which had shallower bodies (Fig. 9a). Comparing the body shapes between temperatures, the shallower bodied fish have some differences in the curvature of their bodies, 20°C fish appear straighter whereas 24°C and 28°C have a slight dorsal curvature (Fig. 9a). Conversely, the 20°C fish in the deeper bodied images show a slight dorsal curvature whereas the 24°C and 28°C fish are a bit straighter (Fig. 9b).

The mixed effects model showed that temperature did not have a significant effect on PC2 when the log of centroid size and every cross were considered (Temperature: x^2=3.15, DF=2, P-value=0.207) (Table 3). However, there was an effect when the crosses with small sample sizes were taken out as well as an effect between PC2 and size (Temperature: x^2=6.44; DF=2, P-value=0.0400; Size: x^2=86.2; DF=1; P-value=2.2E-16) (Table 4). When plotted, PC2 follows similar patterns between each temperature treatment where when size increases every temperature treatment follows a similar nonlinear path to a higher PC2 score, and eventually a deeper body. However, in the crosses with small sample sizes are taken out 28°C fish tend to have an overall deeper body than fish in both the 24°C and 20°C treatments. There is a slight decrease in PC2 from 2.0 to 2.5 centroid size, resulting in thinner bodied fish, then as the fish grow larger from a centroid size near 2.5, they develop deeper bodies (Fig. 10a and 10b).

Bone Development:

Sequence of Bone Development

For the graph of skeletal ossification timing, skeletal structures were placed in the order in which they ossified based on the average across temperature treatments (Fig. 12). The order of ossification is: (1) cleithrum, (2) first precaudal vertebra, (3) first neural spine, (4) dentary, (5) premaxilla, (6) Weberian apparatus, (7) first rib, (8) last neural spine, (9) last caudal vertebra, (10) first hypural, (11) last haemal spine, (12) first caudal fin ray, (13) first haemal spine, (14) last rib, (15) last caudal fin ray, (16) last hypural, (17) first dorsal fin ray, and (18) first anal fin ray. Fifteen of the eighteen bone structures had the smallest size of ossification for the 28°C treatment. The last rib, first haemal spine, and last haemal spine had the smallest size for set ossification at 24°C (Fig. 12).

There were many precocious fish exhibiting ossified skeletal structures at sizes that were below what was typical. This was true for every bone structure and in all temperature treatments. Then the number of variable fish begins to decline in the 20°C treatment with less than 10 fish for every structure that ossifies after the first rib. There were relatively large gaps in ossification size among the fish for the skeletal structures ossifying later in development in the 20°C temperature treatment. The number of fish was relatively constant in the 28°C treatment from the cleithrum to first haemal spine, declining for structures that ossified later. The 24°C treatment is the only treatment that has consistently high numbers of variable fish for every skeletal structure from the cleithrum to the first anal fin ray (Fig. 12). There were also some gaps in ossification size between fish for traits ossifying later in the 24°C treatment like the last hypural, first dorsal fin ray, and first anal fin ray (Fig. 12).

The average order in which early and late developing bones ossified remained relatively consistent between temperatures. All three treatment groups had the same six skeletal structures ossify first (cleithrum, first neural spine, first precaudal vertebra, dentary, and premaxilla) with only the 20°C treatment having the first precaudal vertebra and first neural spine in a different order (3^{rd} and 2^{nd}) than the 24 and 28°C treatments (2^{nd} and 3^{rd}). All temperatures had the same three structures ossify last: (18) the first anal fin ray, (17) first dorsal fin ray, and (16) last hypural (Fig. 13). The structures that ossified at intermediate sizes exhibited more variation in developmental order with first rib, last caudal vertebra, first hypural, last haemal spine, first caudal fin ray, first haemal spine, and last rib ossifying in different sequences between temperatures with a difference in magnitude of rank greater than 1 (Table 5). The last caudal vertebra exhibited a particularly large difference in ranks between the 20 and 28°C treatments, with a rank of 6 for the 28°C treatment and 13 for the 20°C treatment. Other relatively large differences in ranks were seen for the last haemal spine between the 20 and 24°C treatments (both rank =9) and the 28°C treatment (rank = 13), the first haemal spine with a difference of 3 ranks between the 20°C treatment (rank=11) and 24°C treatment (rank=14), and the last rib with a difference of 3 ranks between the 24°C (rank=12) treatment and the 20 and 28°C treatments (both ranks=15). Even with these discrepancies of rank between temperature treatments, the ossification ranks had a near perfect association in pairwise comparisons (20 vs. 24 r=0.96; 20 vs. 28 r= 0.94; 24 vs. 28 r=0.95, Table 6a). The overall order of development was significantly concordant among temperature treatments (Kendall's Coefficient of Concordance W, W= 0.96, x^2= 49.09, p-value< 0.001, Table 6b).

Difference in Rank Variation

On average, there is a difference in rank of 1.5 between the temperature treatments. The average difference in rank change did not vary greatly between temperature treatments. The average rank difference for each temperature compared to the average rank was less than 1, with 20 and 24°C having the lowest average (0.59), but not much smaller than 28°C (0.61) (Table 5; Fig. 14).

Size and Development

Contrary with differences in bone ossification order, there were statistical differences in the set standard length of fish when each bone structure ossified between at least two groups. Of the eighteen structures, five of them had p-values less than their adjusted Benjamini-Hochberg value: the cleithrum ($F(2,17)$ df=6.70, p=0.007), first precaudal vertebra ($F(2,16)$ df=7.52, p=0.005), first neural spine ($F(2,14)$ df=4.86, p=0.025), dentary bone ($F(2,13)$ df=6.33, p=0.012), and the last caudal vertebra ($F(2,9)$ df=7.30, p=0.013) (Table 7; Table 8). The skeletal structures that differed significantly among temperature treatments all exhibited divergence between the average set size of ossification between the 20°C and 28°C treatments, while the first precaudal vertebra and dentary skeletal structures also exhibited a significant difference between the 20°C and 24°C treatments (Table 9). These differences are also apparent in the figures. Figure 16 shows that the skeletal structures that ossify first ossify at smaller sizes in the 28°C treatment than in the 20°C treatment, and consistently stay smaller until the end of the developmental series (Fig. 15). This is consistent also with data for the smallest size of ossification, which shows that fish in the 28°C and 24°C treatments exhibit ossification of skeletal structures at a smaller size than the 20°C treatment fish (Table S3 and Fig. S1).

The coefficient of variation for the size of set bone ossification varied significantly among temperature treatments. Fish in the 20°C treatment had the highest variation levels overall (12 of 18 structures) compared to the 24°C (1 of 18) and 28°C (5 of18) treatments (Table 10, Fig. 16). These differences in levels of variation were statistically significant ($F(2, 50)$ df=4.83, p=0.012), specifically between the 20°C and 24°C treatments (p=0.016, 95% C.I.=8.17,12.48) and between the 20°C and 28°C treatments (p=0.038, 95% C.I.=8.67,12.48) (Table 11).

Discussion:

The main goal of this study was to examine how temperature affects the body shape and ossification of skeletal structures during the early stages of development in *A. mexicanus*. Temperature is a critically important environmental variable that influences many aspects of the development of ectotherms like most fishes (Jordan, 1892; Georgakopoulou et al., 2007; McDowall, 2008; Barriga et al., 2013; Ramler et al., 2014; Ackerly and Ward, 2015; Reyes and Aguirre, 2019). Reyes and Aguirre (2019) showed that temperature had a strong effect on vertebral phenotypes and body shape in *A. mexicanus.* Consequently, I hypothesized that temperature variation will have a significant effect on the pattern and rate of development of the axial skeleton and body shape in *A. mexicanus*. Temperature variation is known to effect developmental timing, with cooler temperatures slowing development whereas warmer temperatures increase developmental rates (Mabee et al., 2000; Morin-Kensicki et al., 2002). Less is known about the effects of temperature on morphology when differences in developmental rate are accounted for.

I found inconclusive results regarding body shape. Shape differences were seen due to differences in larval stage development. These differences were small when comparing the differences between temperatures, and only statistically significant when certain sets of crosses were compared. For the axial skeletal development, I found that bone structures ossify in near perfect concordance despite differences in developmental temperature, with bones not differing significantly in the order of development. Size of ossification, however, differed significantly among temperature treatments such that fish in the 28°C treatment exhibited ossification in some bones (e.g., cleithrum, first precaudal vertebra, first neural spine, dentary bone, and the last caudal vertebra) at a smaller size than the 20°C. Differences in coefficient of variation between

temperature treatments were also observed. The 20°C saw the most variation in set size of ossification among crosses for 12 of the18 bone structures and had a significantly higher variation when compared to 24°C and 28°C treatments. Below I discuss these results and their possible implications in more detail.

Body Shape Variation:

There are several studies that have looked at how temperature affects the body shape of fishes (McDowall, 2008; Barriga et al., 2013; Morris et al., 2018; Reyes and Aguirre, 2019). Ectotherms are particularly influenced by their surrounding temperature, which can alter their metabolism thus modifying the developmental timing of different tissues and organs (Mabee et al., 2000; Hunt von Herbing, 2002; Georgakopoulou et al., 2007; Barriga et al., 2013; Ramler et al., 2014). Temperature modified the developmental of body shape in my study. The body shape of fish in the 20°C treatment developed much more slowly than that in the other temperature treatments (Fig. 4). What took the 24°C and 28°C fish approximately 30 days to develop, took roughly 60 days in the 20°C treatment.

Although temperature had a large effect on the rate at which the body developed, body shape had an inconsistent result once the differences in developmental rate was accounted for. Body shape was not significantly affected by temperature along PC1. PC1 is more closely associated with early and late larval development, such that specimens varying along this axis present more juvenile or adult body shapes per unit body size. The change in shape due to development is expected to appear because body shape changes through ontogeny are expected.

For PC2, crosses 6, 8, 9, and 10 with the largest sample sizes were significantly affected by temperature, but this effect disappeared when analyzing all crosses. Variation along PC2 was

more closely associated with body depth variation. Differences in body depth in fish developing at different temperature were also expected from previous studies (Morin-Kensicki et al., 2002; Bird and Mabee, 2003; McDowall, 2008; Barriga et al., 2013; Morris et al., 2018; Reyes and Aguirre, 2019). Many fish experience changes in body depth due to temperature, with fish developing at cooler temperatures often having elongated thinner bodies, and fish developing at warmer temperatures producing shorter deeper bodies (McDowall, 2008; Barriga et al., 2013; Morris et al., 2018; Reyes and Aguirre, 2019).

Fishes employ many different strategies for swimming and these strategies change with differences in temperature, viscosity, and salinity (Sanderson and Kupferberg, 1999; Hunt von Herbing, 2002; Barriga et al., 2013; Yu et al., 2018). Fishes are not only affected by these factors once they reach juvenile or adult stages, but they are also affected at the larval stage, and differences in body shape have many potential functional implications (Sanderson and Kupferberg, 1999; Hunt von Herbing, 2002; Georgakopoulou et al., 2007). Longer and thinner bodies in cooler water temperatures could be due in part to changes in development that led to improved swimming performance in cold water which has higher viscosity compared to warmer water. Larval fish swim, albeit less than juveniles and adults, but this change in viscosity could be a factor of temperature that leads to changes in body shape needed for different swimming strategies (Sanderson and Kupferberg, 1999; Hunt von Herbing, 2002; Georgakopoulou et al., 2007). For example, Hunt von Herbing (2002) found that temperature induced effects on viscosity lead to more than 40% of the variation in routine and burst swimming speeds in herring larvae. Length and absolute swim speed are positively correlated in fishes, meaning that longer fish can cover more distance per time, whereas with relative swim speed (body lengths per time) smaller fish tend to have higher relative swim speeds (Sanderson and Kupferberg, 1999; Hunt

von Herbing, 2002; Barriga et al., 2013; Yu et al., 2018). However, at lower temperatures, the relationship between relative swimming speed and fish size disappears. The thinner bodies of *A. mexicanus* developing at colder temperatures may help reduce the drag associated with swimming in more viscous environments.

The physical changes that water temperature differences induce suggest a possible direction for future studies. Studying fish swimming performance and its correlation to body shape from larval to adult sizes could lead to further understanding of why body shape changes in different ways over ontogeny in different fish species (Sanderson and Kupferberg, 1999; Hunt von Herbing, 2002; Georgakopoulou et al., 2007). Perhaps in more viscous water (colder water), a longer, thinner body would be more beneficial for their swimming performance, leading to a greater ability to escape predators and catch prey (Sanderson and Kupferberg, 1999; Hunt von Herbing, 2002; Barriga et al., 2013; Yu et al., 2018). If heritable, they could pass on their adaptive plasticity to the next generation.

Bone Development:

I predicted that the more extreme temperature treatments would result in different sizes of ossification, and my data support this. Fish in the 28°C treatments ossified at a significantly smaller standard length than the 20°C treatment in five structures (i.e., cleithrum, first precaudal vertebra, first neural spine, dentary bone, and the last caudal vertebra). The 28°C treatment also had significantly smaller size of development in the first precaudal vertebra and the dentary bone when compared to the 24°C. The differences in set standard length of bone ossification show that there are some differences between size and ossification for some, but not all bone structures. Of the skeletal structures that ossified significantly differently at different temperatures, four of the

five structures were the first structures to ossify. When adding in the two structures with the next smallest P-values for different sizes of ossification (i.e., premaxilla and the Weberian apparatus), they made up the first six structures that ossify. This suggests that the skeletal elements most likely to exhibit variation in the size of ossification are the first ones to develop.

The sequence of bone development was a near perfect 1 for Kendall's Coefficient of Concordance W. This result is not surprising because previous studies found that interspecific and intraspecific variation in other species of fishes and ectotherms like salamanders and frogs were low (Waddington, 1953; Mabee et al., 2000; Grunbaum et al., 2012). Canalization means that organisms develop similarly regardless of environmental changes and underlying genetic variation, and this is what was seen with bone ossification order. Mabee et al. (2000) and Grunbaum et al. (2012) found that bone ossification was relatively conserved in fishes as well. In newly hatched Arctic charr (*Salvelinus alpinus*) exposed to different water flow treatments, the ossification of fins remained similar regardless of water flow differences (Grunbaum et al., 2012). Similarly, Mabee et al. (2000) found that regardless of temperature treatment for *Danio* and a few other bony fish species examined (e.g., *Betta, Oryzias,* and *Barbus)*, bone ossification order was well conserved. In my study, the variation in developmental timing for *A. mexicanus* was small, with only an average of 1.5 ranks in the differences between ossification order between temperature treatments.

Although there was no statistical difference in the order of ossification between temperature treatments, there was statistical differences in size of ossification and coefficient of variation between the 20ºC and the 24 ºC and 28 ºC treatments. Similarly, Grunbaum et al. (2012) found that fish exposed to faster water currents ossified their bones at smaller sizes than fish exposed to slower water currents. Arctic charr have also shown to ossify their axial skeleton

at smaller sizes compared to charr that do not develop in streams (Grunbaum et al., 2012). This is noteworthy because there was variation seen in the size of cranial ossification in the 28°C treatment while also conserving bone ossification order as seen by Grunbaum et al. (2012). Perhaps there is a developmental advantage to ossify certain bones at smaller sizes in warmer temperatures and streams for fishes. The last four bones that were significantly different the first precaudal vertebra, first neural spine, last caudal vertebra, and the dentary bone. All four of these skeletal structures are associated with the vertebral column and swimming, with differences between the 20ºC and 28ºC could possibly be associated with swimming performance in different temperatures and viscosities and with the mechanosensory lateral line system to help with predator and prey detection (Bird and Webb, 2014). Beat and glide swimming, in which larvae beat the tail once and glide, is commonly employed by larval fish (Sanderson and Kupferberg, 1999). Hunt von Herbing (2002) hypothesized that swimming may be a more viable option for larvae in warmer waters than cooler waters, which could possibly explain why the smaller 28ºC develop some spinal structures at a smaller size than the 20ºC fish.

Heterochrony (change of developmental time) is an important aspect of skull development in nearly all bony organisms (Boughton et al., 1991; Bird and Webb, 2014). This is important to consider in this study because fish in the 28°C had one bone associated with the jaw ossify at significantly smaller standard lengths, the dentary bone. With earlier and faster development, these larval fish lose their yolk sacs quicker and would need a more precise way of feeding. Larval fish must consume 50% of their body weight each day for survival after exhausting their yolk, so fish with higher metabolisms and developmental rates will need to start consuming food more quickly than fish developing more slowly (Sanderson and Kupferberg, 1999). The necessity for fish to develop their jaw at different rates has been documented in other

fish. Boughton (1991) found that needlefish experience heterochrony in both body size and jaw morphology within species and between different species. There is substantial variation in when the lower jaw and the upper jaw develop to extend into the needlelike morphology for different feeding strategies in different environments. Heterochrony of jaw development allows fish to use different feeding strategies that are beneficial in different environments. This allows fish to adapt accordingly to their environment depending on food ability during ontogeny (Boughton et al., 1991). In my study, fish developing in warmer temperatures could benefit from the earlier development of the jaw to feed more efficiently at smaller sizes.

Another aspect to consider with skeletal development in the cranium is that the skull is associated with tissues in the neural crest (Bird and Webb, 2014; Powers et al., 2018). The neural crest is involved with the development of the lateral line, a "touch at a distance 6th sense," that is very important in fishes for escaping predators, looking for food, and schooling (Powers et al., 2018). It has also been hypothesized that neuromasts associated with the lateral line are responsible for early appearance of osteoblasts, which deposit bone, or vice versa, where ossification points recruit lateral line primordia towards them (Powers et al., 2018). Developing these traits at smaller sizes for 28°C treatments may be important because fish could potentially need to swim towards prey and away from predators at a smaller size in warmer waters (Sanderson and Kupferberg, 1999; Hunt von Herbing, 2002; Barriga et al., 2013; Yu et al., 2018).

Limitations:

There are some limitations to my study that should be noted. There was significant variation in the number of surviving fry produced among crosses such that the first three crosses

(1, 3, and 5) had small sample sizes for the 28ºC and 24ºC treatments. Cross 6 also had inconsistent numbers of fish for each temperature treatment. This may have led to the inconsistent results seen for the mixed effects model with PC2 seeing significant results only when crosses 1, 3, and 5 were excluded. Sample sizes were larger for crosses 8, 9, and 10. It would be interesting to see if the results would change significantly if sample sizes were larger and more crosses were used.

Another limitation in my study was having gaps in the ossification sequences seen in the 20°C and 24°C treatments. Larger sample sizes within crosses would have resulted in smaller gaps in the developmental series, which may have resulted in more detailed data for the analyses. I also saw large sized fish with no bones ossified in cross 6, but fish in other crosses of similar sizes were completely ossified. Therefore, it is possible that something happened with the staining protocol of those fish, that left data out of the analysis, or that biological family had a strong genetic component to timing in ossification.

Dissolved oxygen levels differ in water depending on what the temperature is. Cooler temperatures can hold more dissolved oxygen than warmer water temperatures can (Frazier et al., 2001; Marks et al., 2012; Remen et al., 2015). Oxygen levels have been shown to affect metabolism and growth in organisms. Perhaps either accounting for oxygen levels to eliminate another variable could be something to do in a future experiment, or possibly adding oxygen levels as a variable to assess and analyze would lead to more information in the results that were found.

Another factor that could have affected the results was that all the fish were fed the same amount of food regardless of temperature. However, different temperatures in nature could have different levels of resources for fish to consume, thus potentially altering the final shape outcome

of the fish. A future study examining at what point in development temperature makes irreversible physical changes would be interesting. If fish were reared at different temperatures, then moved to the same temperature days later, or even the next day, would similar changes be observed in this study?

Conclusions:

Temperature had an effect on the size in which skeletal elements ossified and the body shape, specifically body depth, changed during the larval and juvenile stages in *A. mexicanus.* These changes due to temperature are important to study, because as climates and habitats change, the ability of organisms to adapt to said changes will affect the probability of their survival. Going forward, it would be interesting to examine how these changes in body shape and size of bone ossification could affect the swimming ability, escape performance, and feeding habits of *A. mexicanus* (Hoke et al. 2012). The benefits or costs of ossifying structures at smaller or larger sizes than normal are unclear. There have been studies of how *A. mexicanus* escapes predators (Hoke et al., 2012), but none have examined how phenotypic plasticity related to temperature variation affects swimming performance, and if the benefits of temperature induced plasticity are temperature specific. Understanding whether the temperature-induced phenotypic changes documented for larval and juvenile *A. mexicanus* in this study affect biological fitness is an important direction for future study.

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Tables and Figures:

Table 1 PC1 Linear Mixed Model Fit by Maximum Likelihood

Random Effects	Variance	Standard Deviation	% Variance
Cross	1.25E-04	0.011	27%
Residual	9.04E-04	0.030	73%
Fixed Effects	**Estimate**	**Standard Error**	**t-value**
Intercept (20°C)	-0.274	0.0102	-26.8
Ln Centroid Size	0.100	0.00345	29.1
24°C	-0.266	0.00315	2.39
28°C	-0.270	0.00338	1.28

Likelihood Ratio Test PC1

Models	AIC	Chi Squared	p-value
Full	-2152	-	-
Null Temperature	-2151	5.79	0.055
Null Size	-1651	503.8	2.20E-16

Likelihood table of PC1 with all crosses

Table 2 Reduced PC1 Linear Mixed Model Fit by Maximum Likelihood

Random Effects	Variance	Standard Deviation	% Variance
Cross	1.29E-04	0.0114	27%
Residual	9.17E-04	0.0303	73%
Fixed Effects	**Estimate**	**Standard Error**	**t-value**
Intercept (20°C)	-0.267	0.0125	-21.3
Ln Centroid Size	0.101	0.00410	24.7
24°C	-0.263	0.00393	0.874
28°C	-0.269	0.00395	-0.304

Likelihood Ratio Test PC1

Models	AIC	Chi Squared	p-value
Full	-1445	-	-
Null Temperature	-1448	1.43	0.490
Null Size	-1094	353	< 2.2e-16

Likelihood table of PC1 with crosses 6, 8, 9, and 10

Table 3 PC2 Linear Mixed Model Fit by Maximum Likelihood

Random Effects	Variance	Standard Deviation	% Variance
Cross	1.29E-05	0.00359	12%
Residual	7.63E-04	0.0276	88%
Fixed Effects	**Estimate**	**Standard Error**	**t-value**
Intercept (20°C)	-0.0981	0.00860	-11.4
Ln Centroid Size	0.0361	0.00314	11.5
24°C	-0.0987	0.00288	-0.221
28°C	-0.0934	0.00307	1.54

Likelihood Ratio Test PC2

Models	AIC	Chi Squared	p-value
Full	-2252	-	-
Null Temperature	-2253	3.15	0.207
Null Size	-2138	117	2.2E-16

Likelihood table of PC2 with all crosses

Table 4 Reduced PC2 Linear Mixed Model Fit by Maximum Likelihood

Random Effects	Variance	Standard Deviation	% Variance
Cross	8.79E-06	0.00288	8%
Residual	7.66E-04	0.0277	92%
Fixed Effects	**Estimate**	**Standard Error**	**t-value**
Intercept (20°C)	-0.0999	0.0103	-9.68
Ln Centroid Size	0.0371	0.00374	9.92
24°C	-0.0991	0.00359	0.206
28°C	-0.108	0.00361	2.33

Likelihood Ratio Test PC2

Models	AIC	Chi Squared	p-value
Full	-1516	-	-
Null Temperature	-1514	6.44	0.0400
Null Size	-1432	86.2	2.2E-16

Likelihood table of PC2 with crosses 6, 8, 9, and 10

Table 5 Ranked Average Length of Ossification of Bone Structures at Three Temperatures

	Ranks (row-wise)			Average Rank	Magnitude of Rank
Bone Structures	20°C	24C°C	28°C		
Cleithrum	1	1	1	1	0
First Precaudal Vertebra	2	3	2	2.3	1
First Neural Spine	3	2	3	2.7	1
Dentary	4	4	4	4	0
Premaxilla	5	5	5	5	0
Weberian Apparatus	6	6.5	6	6.2	0.5
First Rib	7	6.5	9	7.5	2.5
Last Neural Spine	9	9	8	8.7	1
Last Caudal Vertebra	13	9	7	9.7	6
First Hypural	9	11	10	10	2
Last Haemal Spine	9	9	13	10.3	4
First Caudal Fin Ray	12	13	11	12	2
First Haemal Spine	11	14	12	12.3	3
Last Rib	15	12	15	14	3
Last Caudal Fin Ray	14	15	14	14.3	1
Last Hypural	16	16	16	16	0
First Dorsal Fin Ray	17	17	17	17	0
First Anal Fin Ray	18	18	18	18	0
Average Difference in Rank	0.59	0.59	0.61		1.5

Bones of *Astyanax mexicanus* listed in approximate order of ossification between temperature with magnitude of rank variation for each bone measured between crosses. The average rank was taken from ranks between crosses. The magnitude of rank (difference between the highest and lowest rank) was taken for each bone structure and compared for between the temperature treatment groups.

Table 6a Spearman's Correlation of Ranks

	20°C	24°C	28°C
20°C			
24°C	0.956 (P < 0.001)		
28°C	0.936 (P < 0.001)	0.952 (P < 0.001)	

Spearman's correlation coefficient for pairwise comparisons of bone ossification ranks of temperatures (n = 16).

Table 6b Kendall's Coefficient of Concordance W

W	0.96
x^2	49.09
p-value	<0.001

Statistics of Kendall's Coefficient of Concordance W comparing rank of bone ossification between temperature treatments.

Table 7 Average Fish Standard Length in millimeters for Set Ossification of Skeletal Elements

Bone Structures	20°C	24C°C	28°C
Cleithrum	**8.70 (7)**	**7.85 (6)**	**6.64 (7)**
First Precaudal Vertebra	**9.02 (7)**	**8.04 (6)**	**7.82 (6)**
First Neural Spine	**9.24 (6)**	**8.03 (6)**	**7.98 (5)**
Dentary	**9.65 (6)**	**8.10 (5)**	**8.02 (5)**
Premaxilla	9.68 (5)	8.49 (5)	8.22 (5)
Weberian Apparatus	9.94 (5)	9.06 (4)	8.41 (4)
First Rib	10.05 (5)	9.06 (4)	8.88 (4)
Last Neural Spine	10.16 (5)	9.24 (4)	8.82 (4)
Last Caudal Vertebra	**10.63 (4)**	**9.24 (4)**	**8.78 (4)**
First Hypural	10.16 (5)	9.29 (4)	9.12 (4)
Last Haemal Spine	10.16 (5)	9.24 (4)	9.97 (4)
First Caudal Fin Ray	10.35 (5)	9.76 (4)	9.43 (4)
First Haemal Spine	10.24 (6)	9.77 (4)	9.96 (4)
Last Rib	11.33 (4)	9.56 (3)	10.46 (3)
Last Caudal Fin Ray	10.84 (5)	11.27 (4)	10.45 (4)
Last Hypural	11.65 (2)	12.05 (3)	10.52 (4)
First Dorsal Fin Ray	12.06 (2)	12.83 (3)	11.93 (3)
First Anal Fin Ray	14.15 (1)	13.46 (3)	12.91 (3)

Average fish standard length in millimeters for set ossification of skeletal elements by temperature treatment. Numbers in parentheses are the number of crosses used to compute the averages.

Table 8 Benjamini-Hochberg P-Value Table

Bone Structures	P-Value	*(i/m)Q*
First Precaudal Vertebra	**0.005**	**0.011**
Cleithrum	**0.007**	**0.022**
Dentary	**0.012**	**0.033**
Last Caudal Vertebra	**0.013**	**0.044**
First Neural Spine	**0.025**	**0.056**
Weberian Apparatus	0.104	0.067
Premaxilla	0.104	0.078
Last Rib	0.122	0.089
Last Neural Spine	0.133	0.100
First Rib	0.151	0.111
Last Hypural	0.153	0.122
First Hypural	0.209	0.133
First Caudal Fin Ray	0.507	0.144
Last Haemal Spine	0.548	0.156
First Anal Fin Ray	0.573	0.167
Last Caudal Fin Ray	0.702	0.178
First Dorsal Fin Ray	0.707	0.189
First Haemal Spine	0.906	0.200

The P-Values of an ANOVA testing for differences in mean size of set ossification for each skeletal element among temperature treatments. The ANOVA was conducted using cross means for each temperature. The last column Benjamini-Hochberg critical value for multiple tests. Skeletal elements for which set size differs significantly with Benjamini-Hochberg adjustment among temperature treatments are in bold.

Table 9 Tukey Post Hoc of Standard Length at Bone Ossification

Temperature	p-values				
Comparisons	Cleithrum	First Neural Spine	First Precaudal Vertebra	Dentary	Last Caudal Vertebra
20°C-24°C	0.33	0.051	**0.026**	**0.031**	0.052
20°C-28°C	**0.0067**	**0.042**	**0.0071**	**0.023**	**0.013**
24°C-28°C	0.12	0.99	0.8	0.99	0.65

Tukey Post Hoc of standard length at bone ossification comparing which treatments saw differences in set size of bone ossification.

Table 10 Coefficient of Variation Computed from the Set Size of Ossification Among Crosses within Temperature Treatments

	Coefficient of Variation		
Bone Structures	20°C	24C°C	28°C
Cleithrum	10.60	8.53	**21.04**
First Precaudal Vertebra	**8.33**	7.34	4.48
First Neural Spine	**12.05**	7.39	4.92
Dentary	**13.09**	7.76	2.14
Premaxilla	**15.07**	11.81	4.72
Weberian Apparatus	**13.45**	8.11	4.70
First Rib	**10.99**	8.11	7.84
Last Neural Spine	**11.06**	5.90	10.83
Last Caudal Vertebra	4.47	5.90	**11.36**
First Hypural	**11.06**	5.18	9.01
Last Haemal Spine	11.06	5.90	**18.23**
First Caudal Fin Ray	11.82	**13.93**	9.09
First Haemal Spine	16.67	14.09	**18.35**
Last Rib	**9.46**	8.26	9.18
Last Caudal Fin Ray	**15.25**	13.66	1.80
Last Hypural	**13.30**	6.94	5.96
First Dorsal Fin Ray	**24.51**	2.59	3.35
First Anal Fin Ray	-	5.64	**9.05**

Variability of ossification between structure and temperature. Bolded numbers are the highest coefficient of variation between the three temperature treatments.

Table 11 Tukey Post Hoc of Coefficient of Variation

Temperature Comparisons	Adjusted p-values
24°C-28°C	0.94
20°C-24°C	0.016
20°C-28°C	0.038

Tukey Post Hoc table showing the adjusted p-values of the coefficient of variation between temperature treatment groups.

Figure 1. Image of a 27 DPF fish with landmarks placed around the body used to analyze body shape. Landmarks missing around body to account for differences in food consumption. Scale bar represents 5mm.

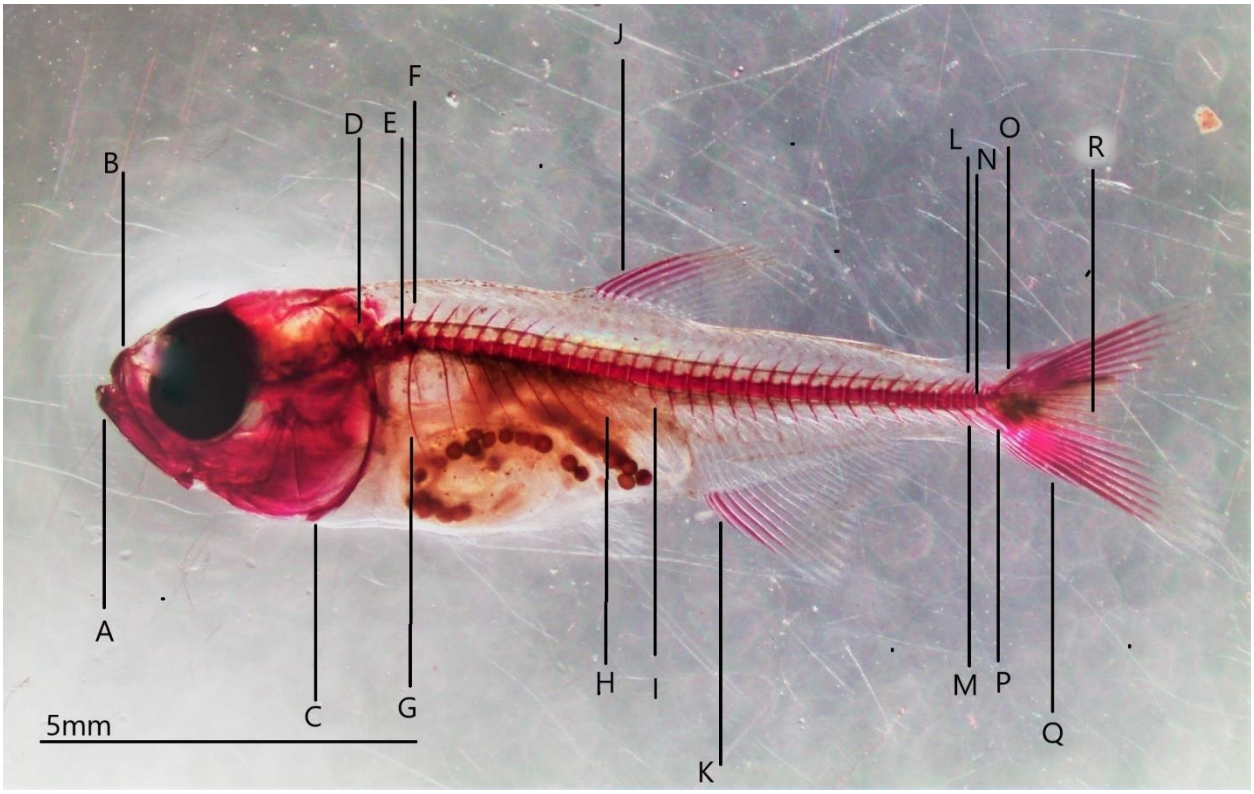


Figure 2. Image of a 21 DPF fish from Cross 8 28°C treatment. Labels are pointing to the bone characters measured for analysis. Scale bar at the bottom is 5mm. a. dentary; b. premaxilla; c. cleithrum; d. Weberian apparatus; e. first precaudal vertebra; f. first neural spine; g. first rib; h. last rib; i. first haemal spine; j. first dorsal fin ray; k. first anal fin ray; l. last neural spine; m. last haemal spine; n. last caudal vertebra; o. last hypural; p. first hypural; q. first caudal fin ray; r. last caudal fin ray.

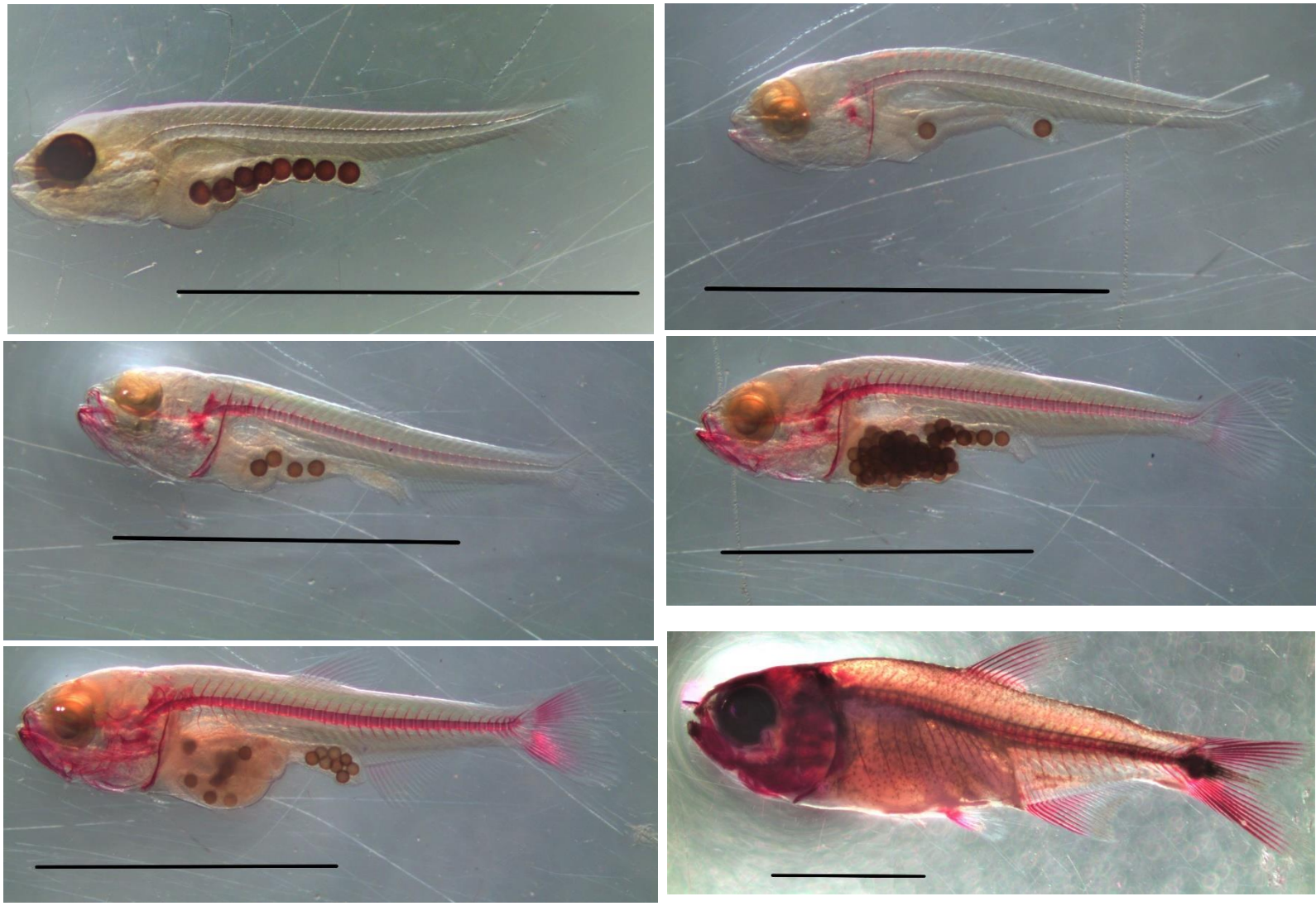


Figure 3. A developmental progression of bone ossification in 24°C fish from cross 8. Fish, from left to right, are: 9DPF, 11DPF, 14DPF, 16DPF, 17DPF, and 35DPF. Scale bars signify 5mm.

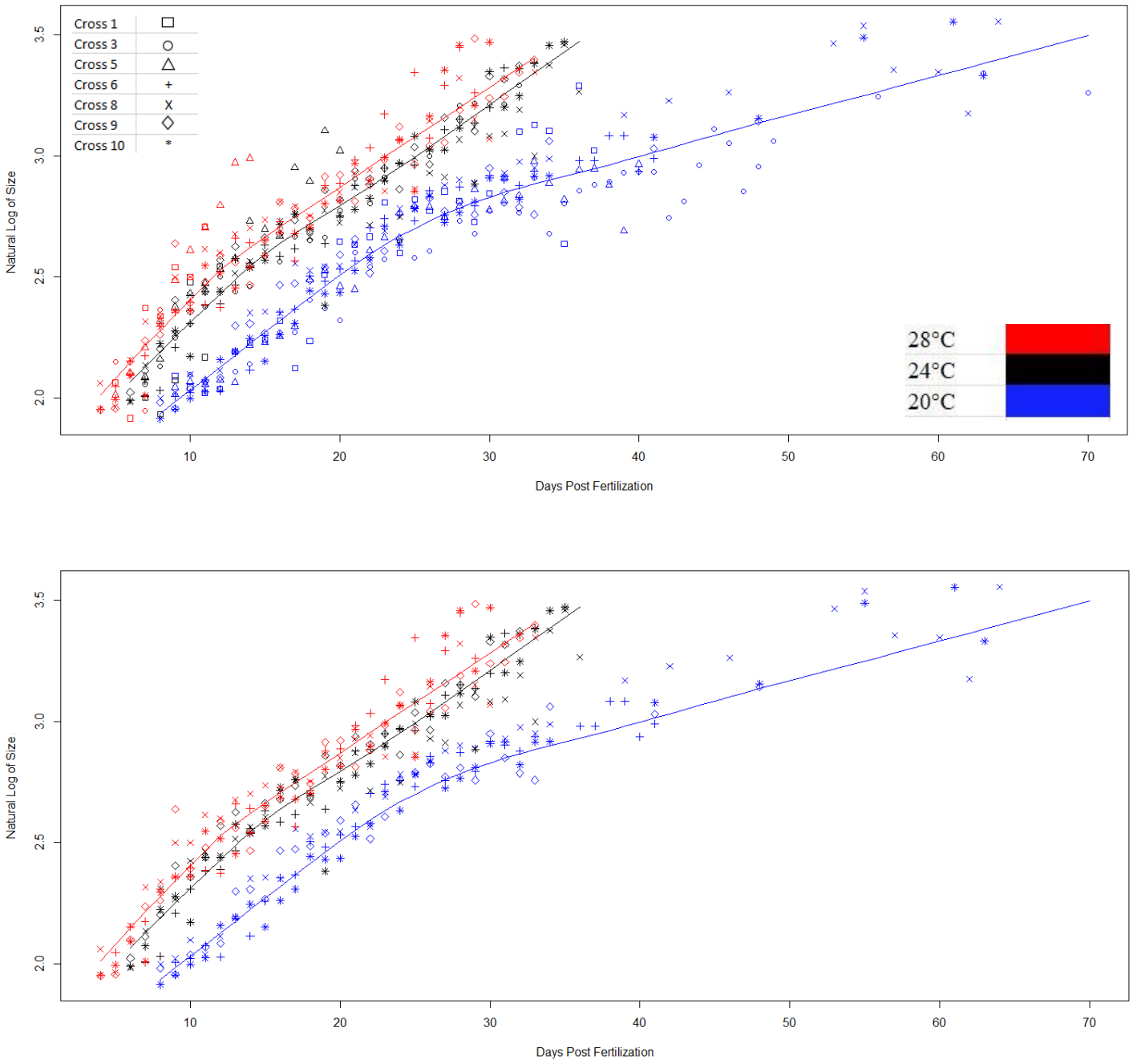


Figure 4. (a) Changes in size over time (days post fertilization) showing the differences in developmental timing between the temperatures and all crosses. Size and age are highly correlated with each other (20°C R^2= 0.878; 24°C R^2= 0.920; 28°C R^2= 0.907). (b) Changes in size over time (days post fertilization) showing the differences in developmental timing between the temperatures and crosses 6, 8, 9, and 10. Size and age are even more highly correlated with each other when the small sample size crosses are removed (20°C R^2= 0.940; 24°C R^2= 0.934; 28°C R^2= 0.904).

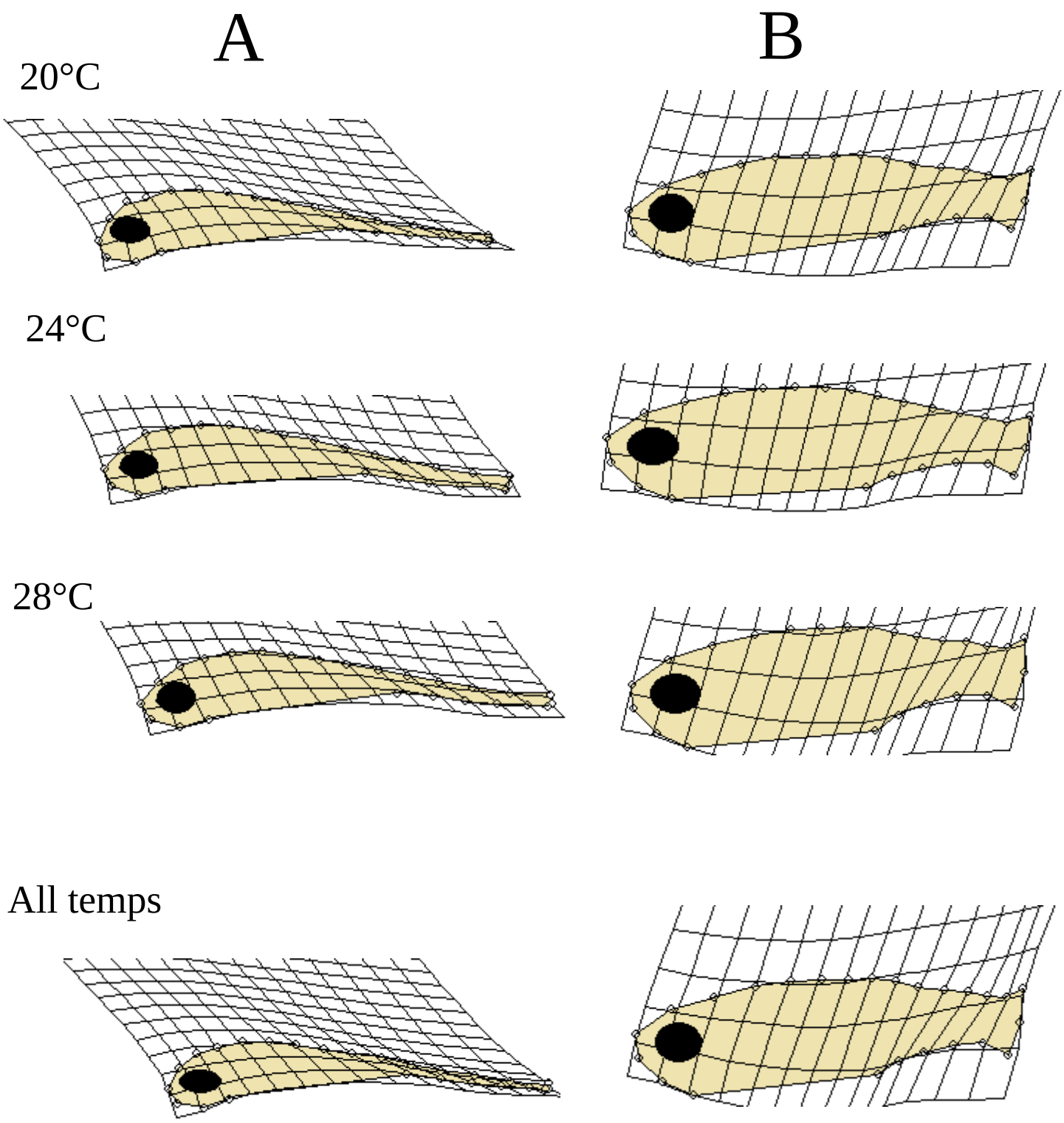


Figure 5. The extreme shapes of body shape of PC1. Smaller fish represent the negative ends (A); larger fish represent the positive ends (B). Each temperature treatment is labeled with its corresponding temperature.

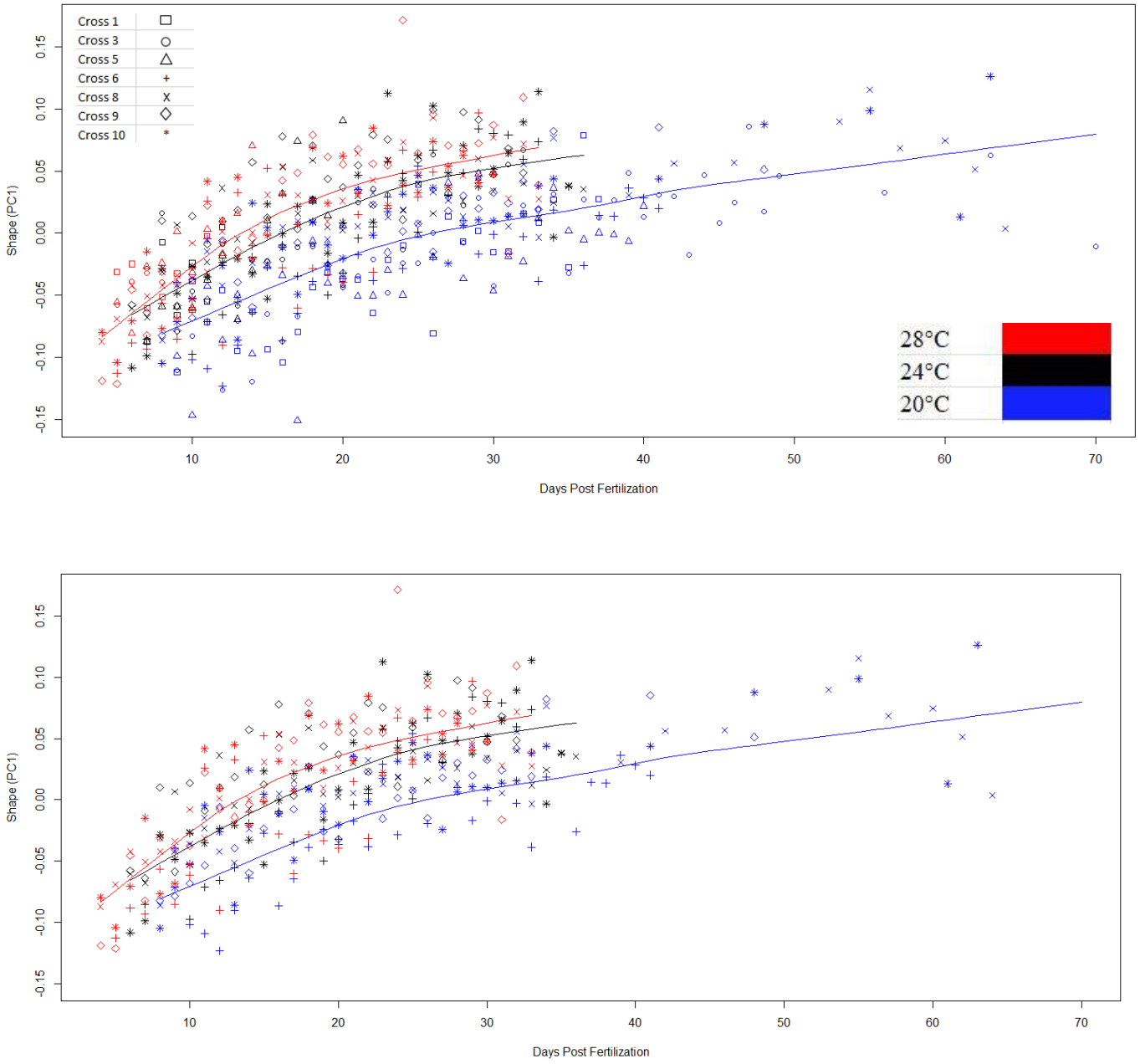
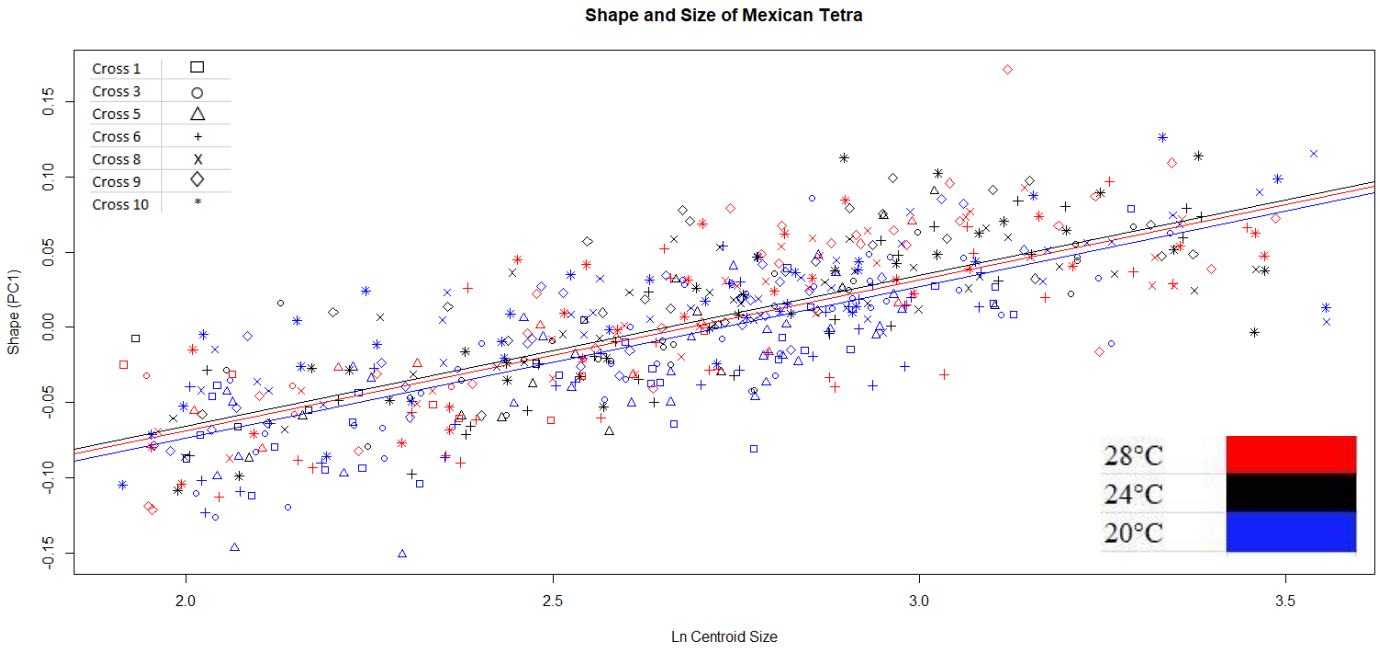


Figure 6. (a) Changes in PC1 over time showing the differences in developmental timing between the temperatures and all crosses. 20°C develops at the slowest rate while 28°C develops fastest with 24°C developing slightly slower than 28°C. PC1 scores are slightly correlated with DPF (20°C R^2= 0.561; 24°C R^2= 0.607; 28°C R^2= 0.684).
(b) Changes in PC1 over time showing the differences in developmental timing between the temperatures of crosses 6, 8, 9, and 10. 20°C develops at the slowest rate while 28°C develops fastest with 24°C developing slightly slower than 28°C. PC1 of crosses 6, 8, 9, and 10 scores are slightly correlated with DPF as well (20°C R^2= 0.611; 24°C R^2= 0.602; 28°C R^2= 0.690).



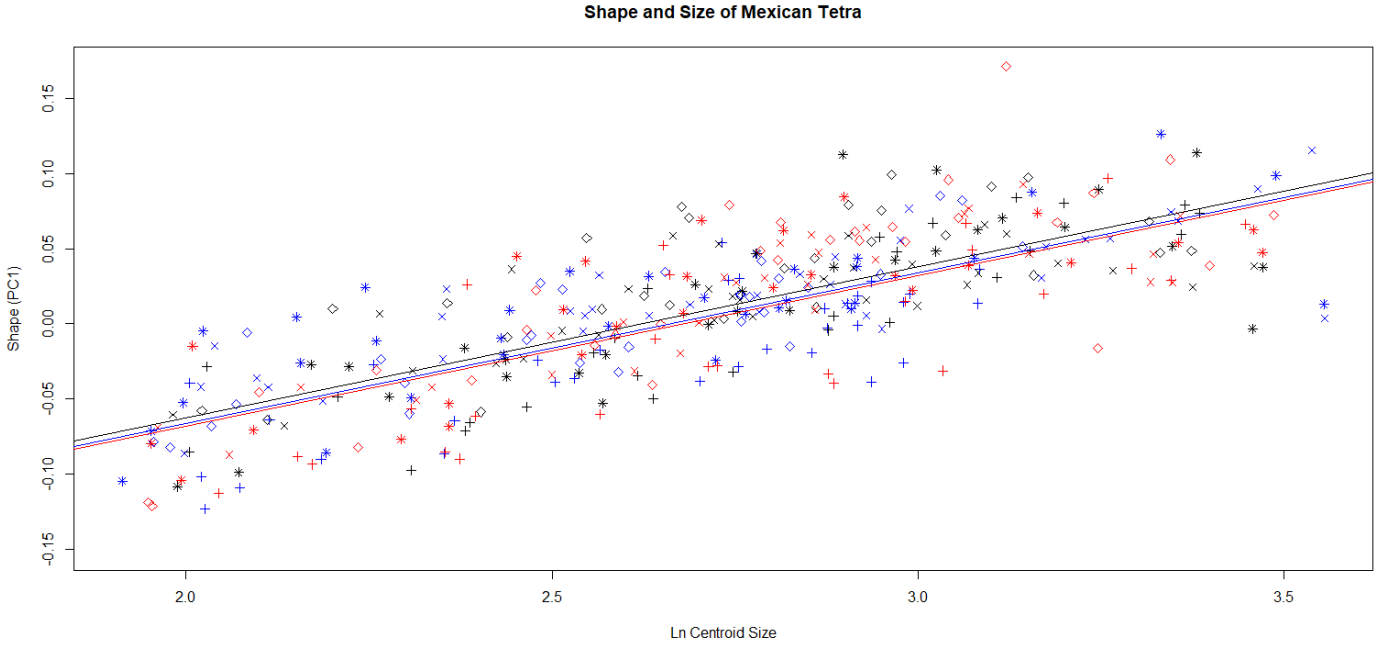


Figure 7. (a) PC1 scores of the Mexican Tetra as the size increases for all crosses. PC1 scores are strongly correlated with log centroid size (20°C R^2= 0.593; 24°C R^2= 0.610; 28°C R^2= 0.627).
(b) Graph of PC1 shape change of the Mexican tetra crosses 6, 8, 9, and 10. As centroid size increases PC1 increases for all temperatures to a more developed fish shape (20°C R^2= 0.615; 24°C R^2= 0.590; 28°C R^2= 0.621).

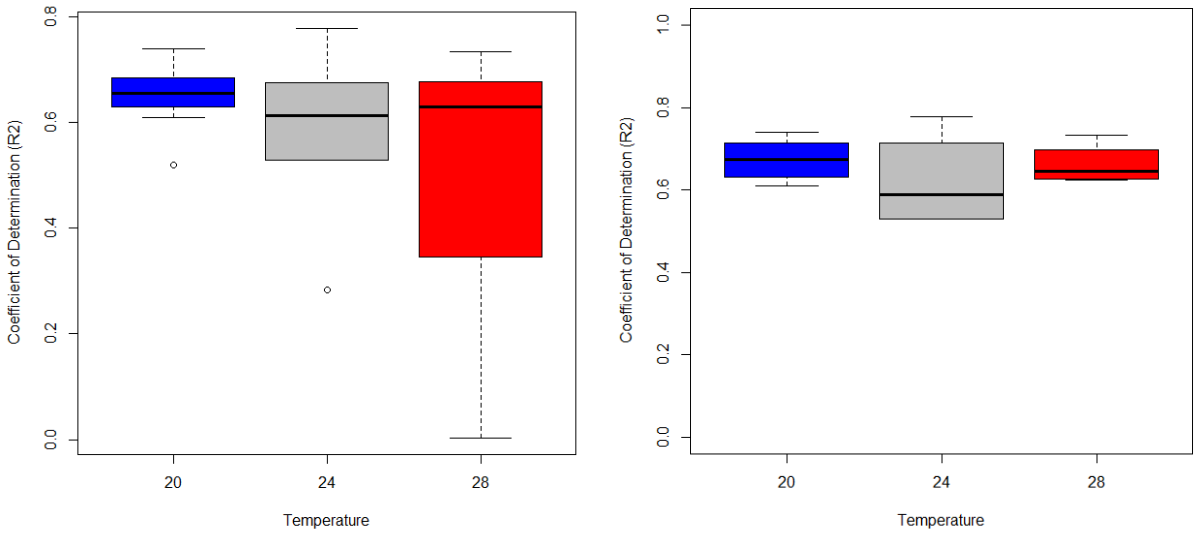


Figure 8. (a) Box plots of R^2 values of PC1 and size of every cross. (b) Box plots of R^2 values of PC1 and size of the full crosses (6, 8, 9, and 10).

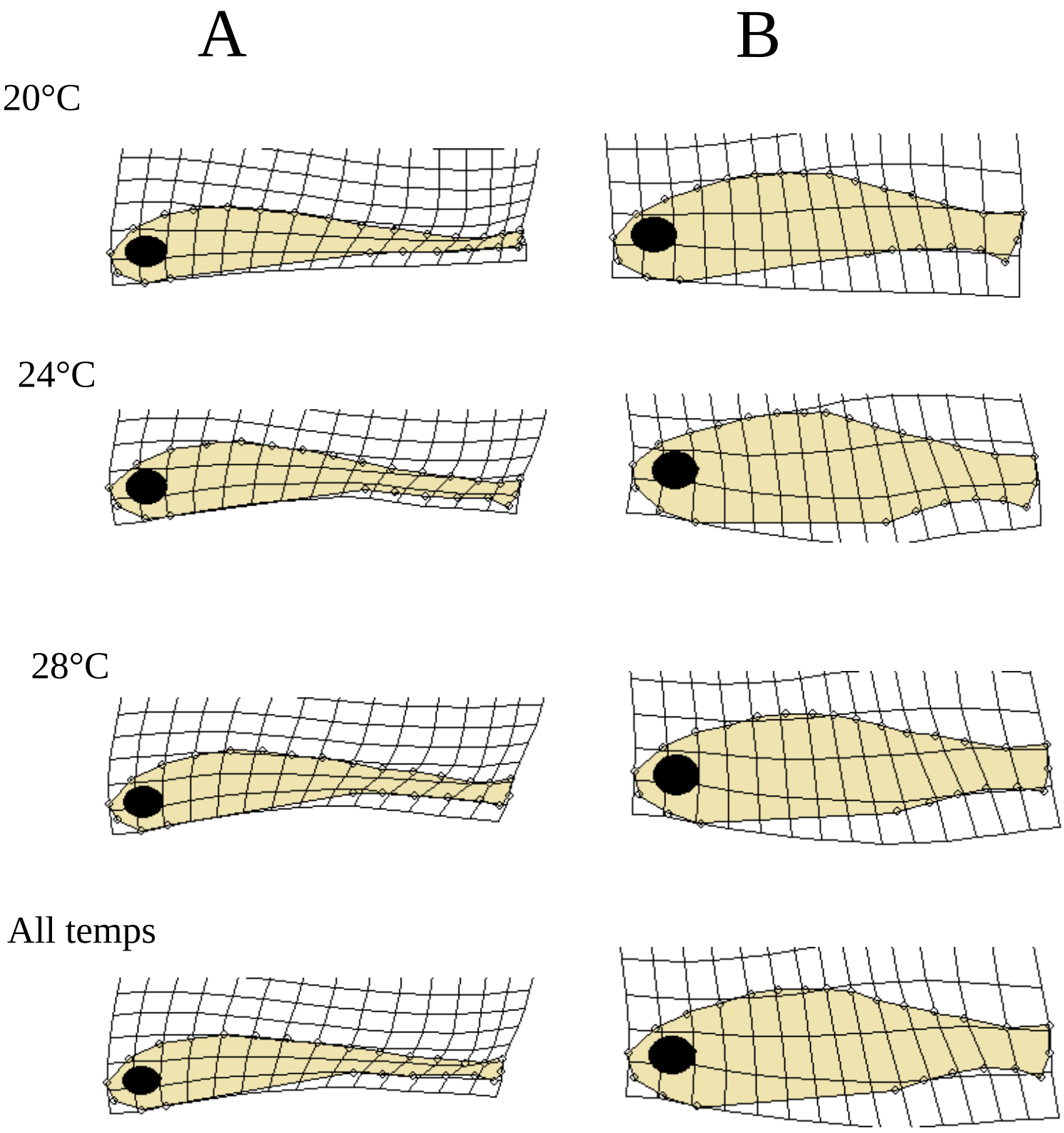


Figure 9. The extreme shapes of body shape of PC2. Thinner bodied fish represent the negative ends (A); deeper bodied fish represent the positive ends (B). Each temperature treatment is labeled with its corresponding temperature.

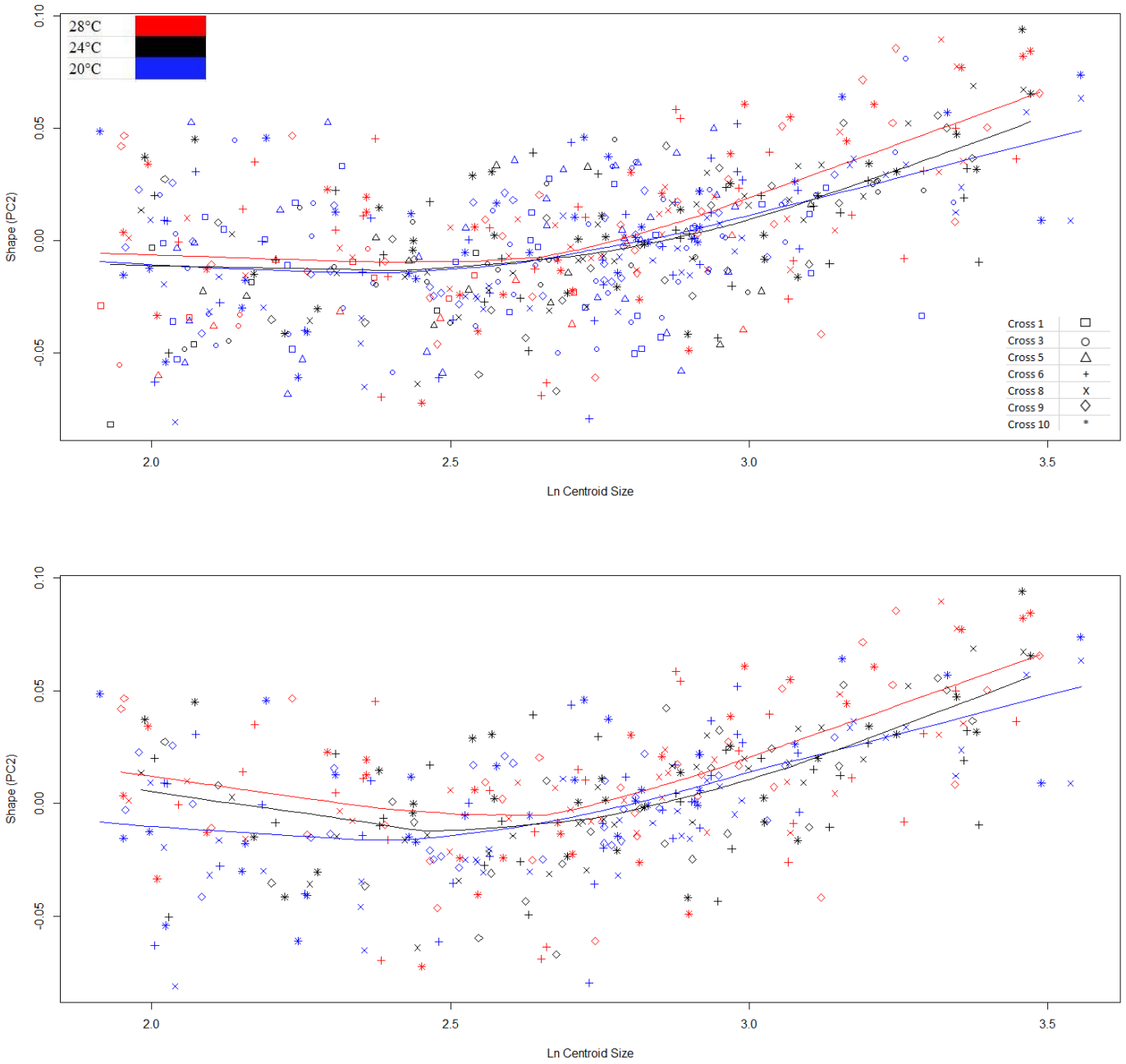


Figure 10. (a) Change in PC2 over the change in size of *A. mexicanus.* As size increases body depth follows a curved path that leads to deeper bodies. (R^2 20°C=0.202; R^2 24°C=0.362; R^2 28°C=0.378).
(b) Change of PC2 over change in centroid size. As size increases body depth follows a curved path that leads to deeper bodies. (R^2 20°C=0.314; R^2 24°C=0.424; R^2 28°C=0.384).

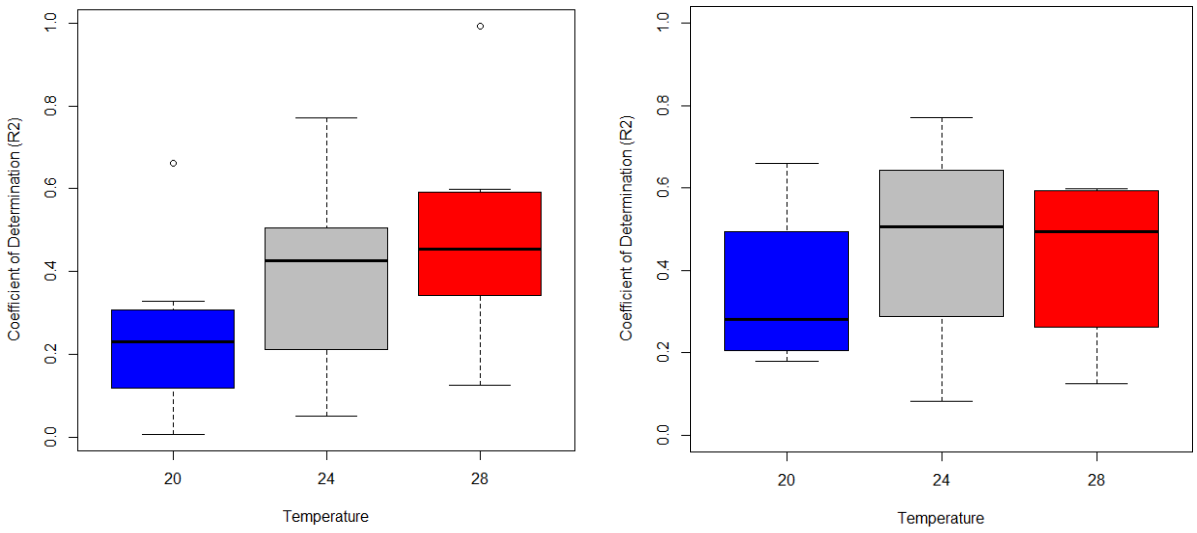


Figure 11. (a) Box plots of R^2 values of PC2 and size of every cross. (b) Box plots of R^2 values of PC2 and size of the full crosses (6, 8, 9, and 10).

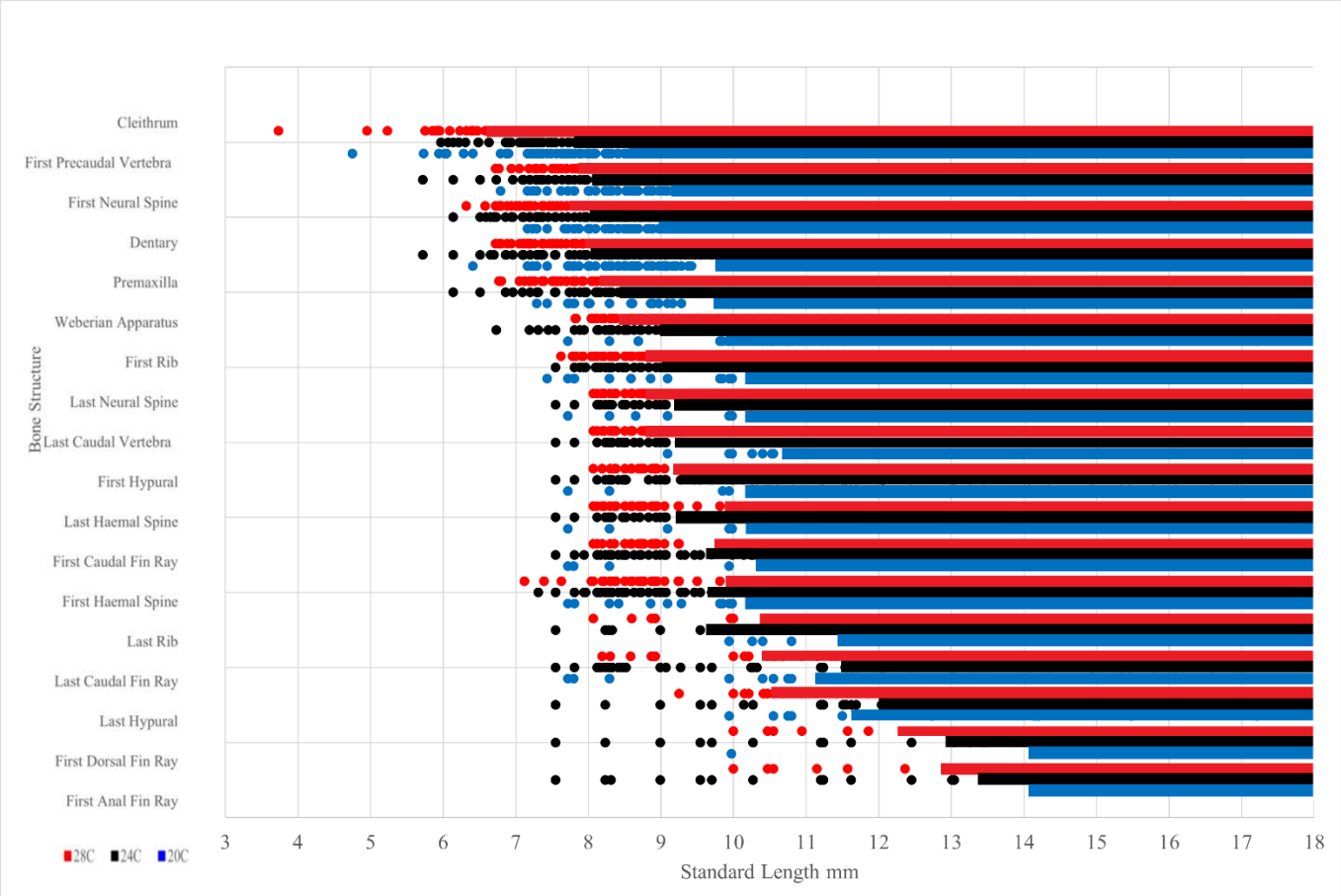


Figure 12. Sequence of average bone ossification for eighteen skeletal structures scored in fish raised at 20, 24, and 28°C. Circles indicate earliest appearances of ossification. Bars represent point after which all specimens exhibited ossification for the structure across all crosses.

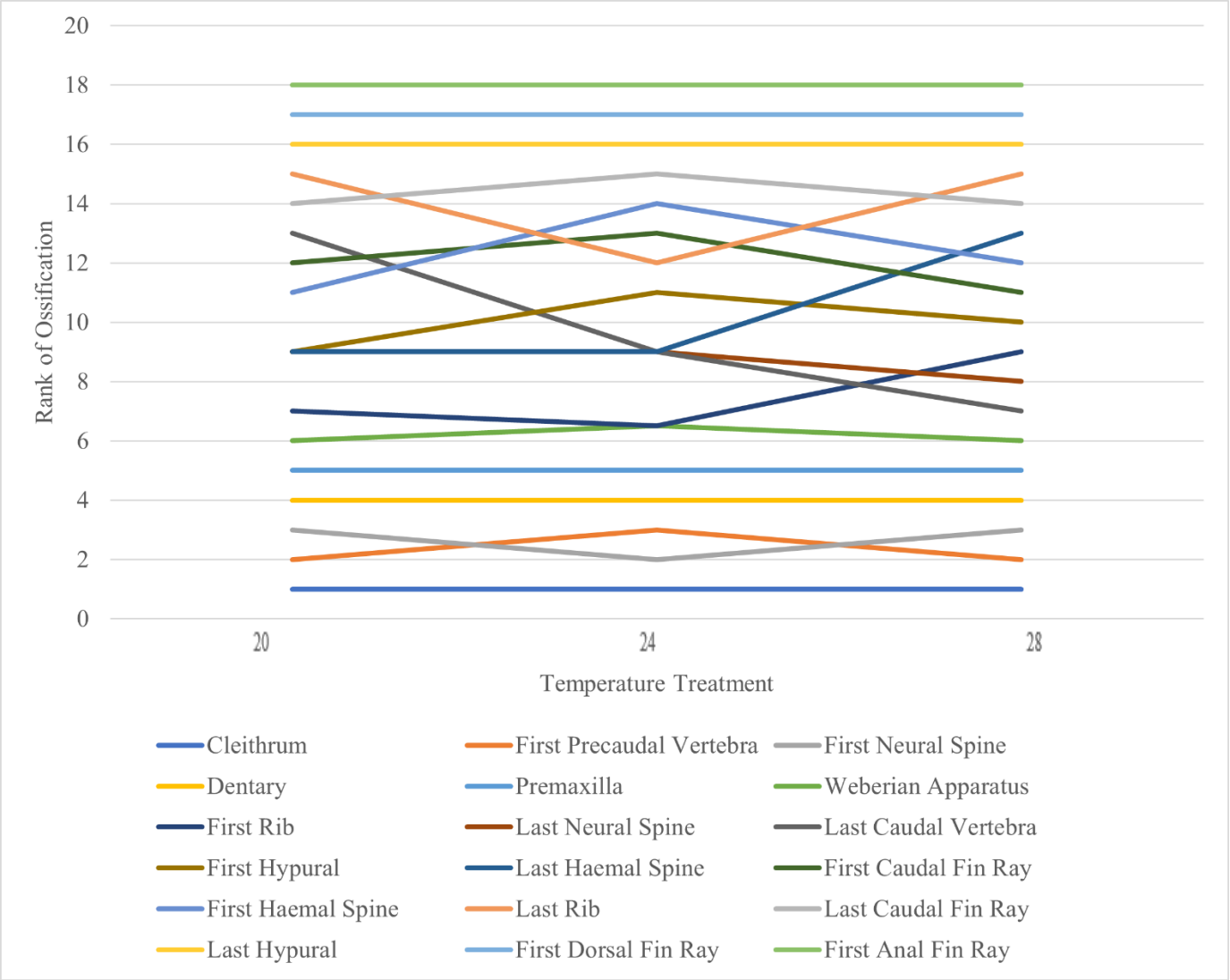


Figure 13. Order of ossification rank between temperatures.

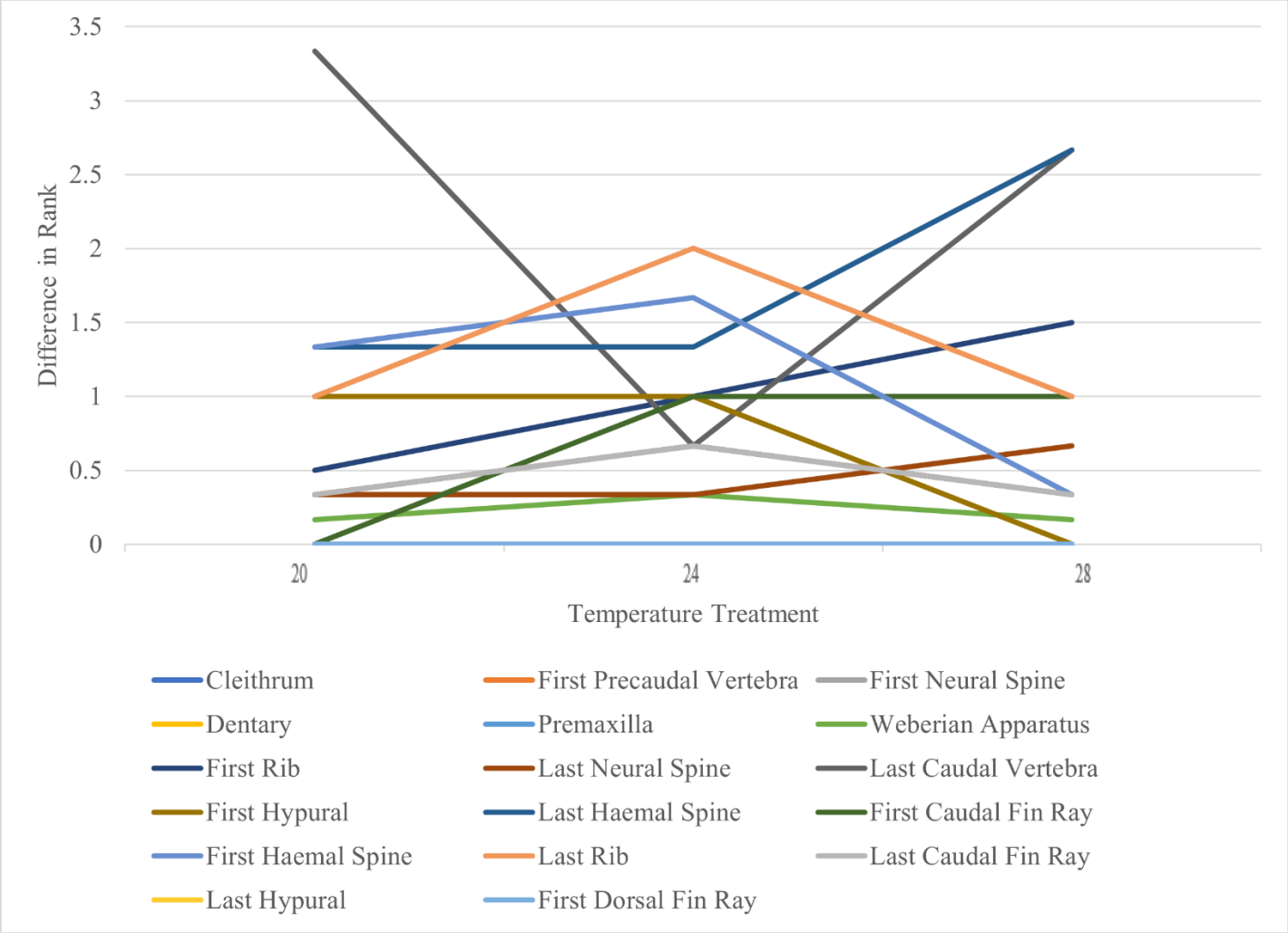


Figure 14. Magnitude of Rank of ossification order rank across crosses and between temperatures comparing the temperature treatments to the average rank.

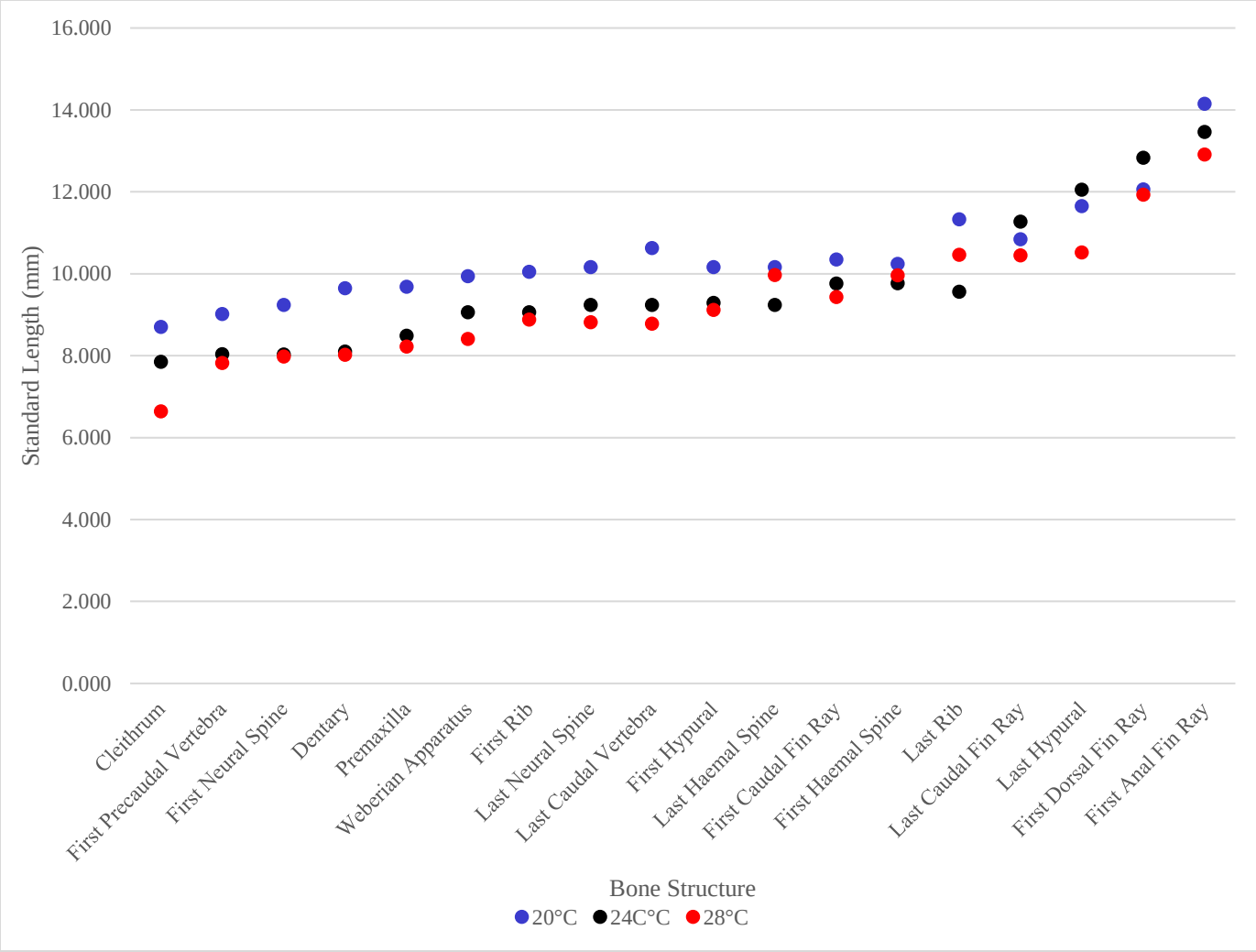


Figure 15. Average Size (mm) at Set Ossification of each skeletal element by temperature.

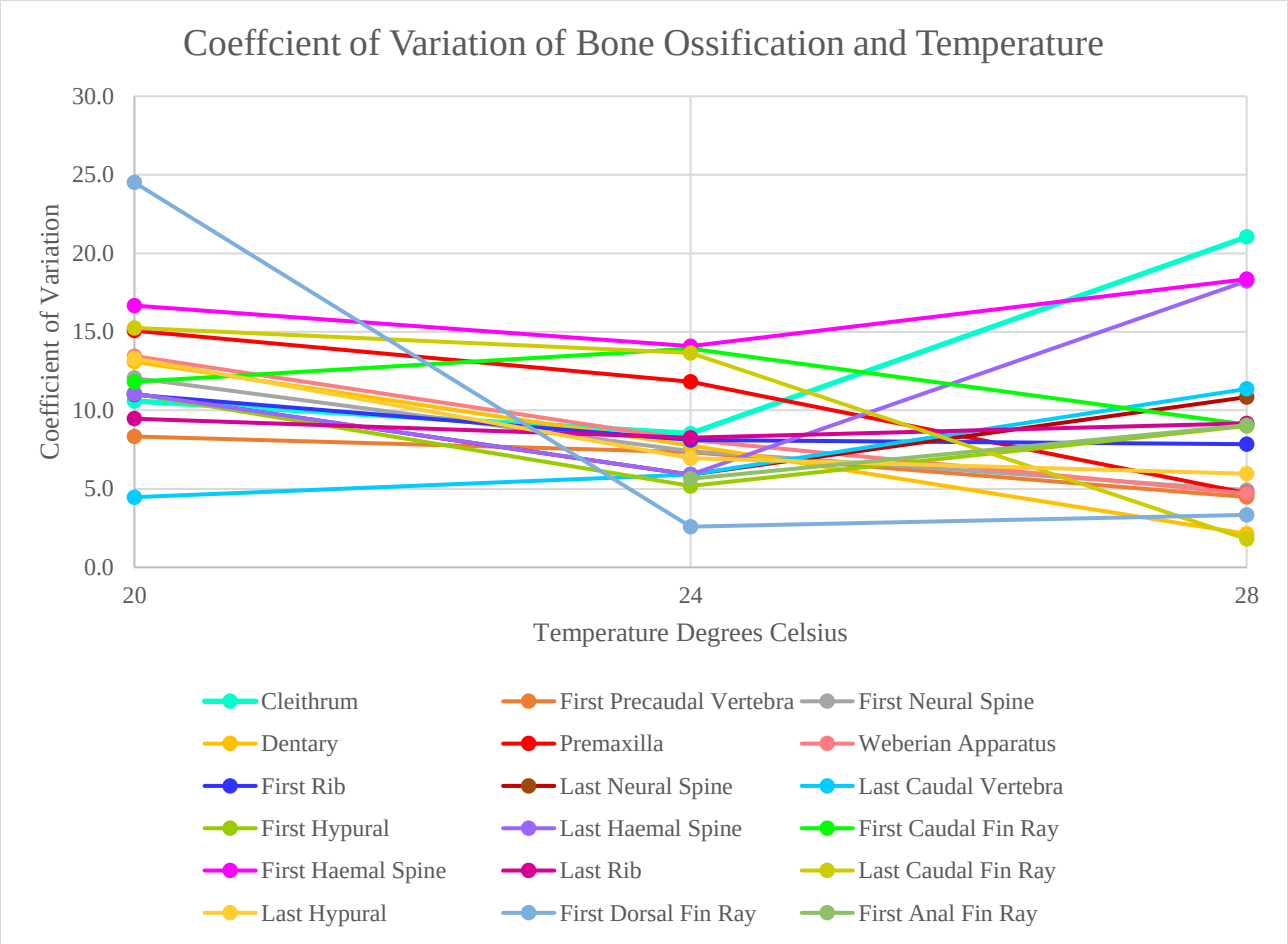


Figure 16. Coefficient of variation of bone ossification between temperatures.

Supplemental Material:

Table S1a	PC1
Cross and Temp	R^2
C1 20°C	0.65
C1 24°C	0.28
C1 28°C	0.0035
C3 20 °C	0.68
C3 24°C	0.61
C3 28°C	0.065
C5 20°C	0.52
C5 24°C	0.70
C5 28°C	0.69
C6 20°C	0.65
C6 24°C	0.77
C6 28°C	0.62
C8 20°C	0.69
C8 24°C	0.53
C8 28°C	0.73
C9 20°C	0.74
C9 24°C	0.52
C9 28°C	0.66
C10 20°C	0.61
C10 24°C	0.65
C10 28°C	0.63

R^2 values of all crosses when looking at PC1 scores and Ln centroid size.

Table S1b	PC1
Cross and Temp	R^2
C6 20°C	0.65
C6 24°C	0.77
C6 28°C	0.62
C8 20°C	0.69
C8 24°C	0.53
C8 28°C	0.73
C9 20°C	0.74
C9 24°C	0.52
C9 28°C	0.66
C10 20°C	0.61
C10 24°C	0.65
C10 28°C	0.63

R^2 values of crosses with large sample sizes when looking at PC1 scores and Ln centroid size.

Table S2a	PC2
Cross and Temp	R^2
C1 20°C	0.0071
C1 24°C	0.34
C1 28°C	0.46
C3 20 °C	0.29
C3 24°C	0.43
C3 28°C	0.99
C5 20°C	0.057
C5 24°C	0.051
C5 28°C	0.29
C6 20°C	0.23
C6 24°C	0.082
C6 28°C	0.13
C8 20°C	0.66
C8 24°C	0.77
C8 28°C	0.60
C9 20°C	0.18
C9 24°C	0.52
C9 28°C	0.40
C10 20°C	0.33
C10 24°C	0.49
C10 28°C	0.59

R^2 values of all crosses when looking at PC2 scores and Ln centroid size.

Table S2b	PC1
Cross and Temp	R^2
C6 20°C	0.23
C6 24°C	0.082
C6 28°C	0.13
C8 20°C	0.66
C8 24°C	0.77
C8 28°C	0.60
C9 20°C	0.18
C9 24°C	0.52
C9 28°C	0.40
C10 20°C	0.33
C10 24°C	0.49
C10 28°C	0.59

R^2 values of crosses with large sample sizes when looking at PC2 scores and Ln centroid size.

Table S3 Average Fish Standard Length in millimeters for First Ossification of Skeletal Elements

Bone Structures	20°C	24C°C	28°C
Cleithrum	6.31 (7)	6.55 (6)	5.46 (7)
First Precaudal Vertebra	8.06 (7)	7.12 (6)	7.11 (6)
First Neural Spine	7.91 (6)	6.983 (6)	7.11 (5)
Dentary	7.55 (6)	6.58 (5)	7.08 (5)
Premaxilla	8.55 (5)	7.11 (5)	7.48 (5)
Weberian Apparatus	9.25 (5)	7.50 (4)	8.23 (4)
First Rib	9.13 (5)	7.80 (4)	8.13 (4)
Last Neural Spine	9.31 (5)	8.29 (4)	8.65 (4)
Last Caudal Vertebra	9.82 (4)	8.28 (4)	8.65 (4)
First Hypural	9.87 (5)	8.28 (4)	8.71 (4)
Last Haemal Spine	9.40 (5)	8.29 (4)	8.65 (4)
First Caudal Fin Ray	10.15 (5)	8.03 (4)	8.77 (4)
First Haemal Spine	9.01 (6)	7.80 (4)	8.25 (4)
Last Rib	11.12 (4)	8.70 (3)	9.04 (3)
Last Caudal Fin Ray	10.63 (5)	8.72 (4)	9.29 (4)
Last Hypural	11.34 (2)	9.69 (3)	10.21 (4)
First Dorsal Fin Ray	12.06 (1)	8.94 (3)	10.93 (3)
First Anal Fin Ray	14.15 (1)	8.94 (3)	10.91 (3)

Average fish standard length in millimeters for first ossification of skeletal elements by temperature treatment. Numbers in parentheses are the number of crosses used to compute the averages.

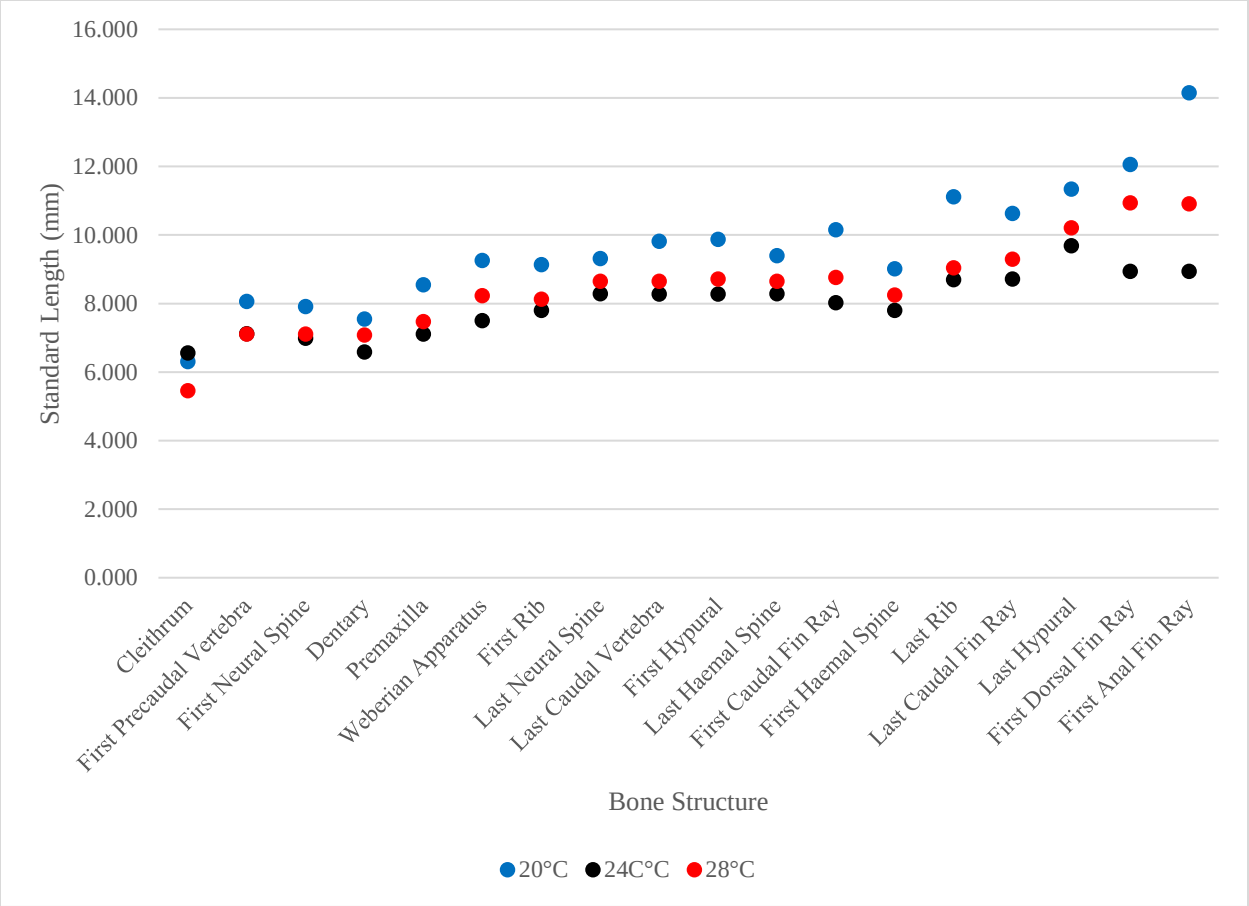


Figure S1. Average Size (mm) at First Ossification of each skeletal element by temperature.