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The effect of rearing temperature on somite formation and skeletal development in the zebrafish, *Danio rerio*

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THE EFFECT OF REARING TEMPERATURE ON SOMITE FORMATION AND
SKELETAL DEVELOPMENT IN THE ZEBRAFISH, *DANIO RERIO*

A Thesis

Submitted

In Partial Fulfillment

of the Requirements for the Designation

University Honors with Distinction

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REARING TEMPERATURE AND DEVELOPMENT IN ZEBRAFISH

This study by: Sarah Jean Freeland

Entitled: The Effect of Rearing Temperature on Somite Formation and Skeletal Development in the Zebrafish, *Danio rerio*

has been approved as meeting the thesis or project requirement for the Designation University Honors with Distinction

Date

Dr. Nathan Bird, Honors Thesis Advisor, Biology

Date

Dr. Jessica Moon, Director, University Honors Program

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INTRODUCTION

The axial skeleton, which consists of the vertebral column and median fins, makes up the largest skeletal region in fishes. The vertebral column forms the longitudinal axis of the body, and is the dominant region of the axial skeleton (Figure 1). During ontogeny, the vertebral

column replaces the embryonic notochord and relies on tight developmental and genetic regulation for proper formation in order to produce the stereotyped segmentation pattern for which the vertebral column is well known (Holley, 2007). The iterative nature of the vertebral column can be traced back to early embryonic

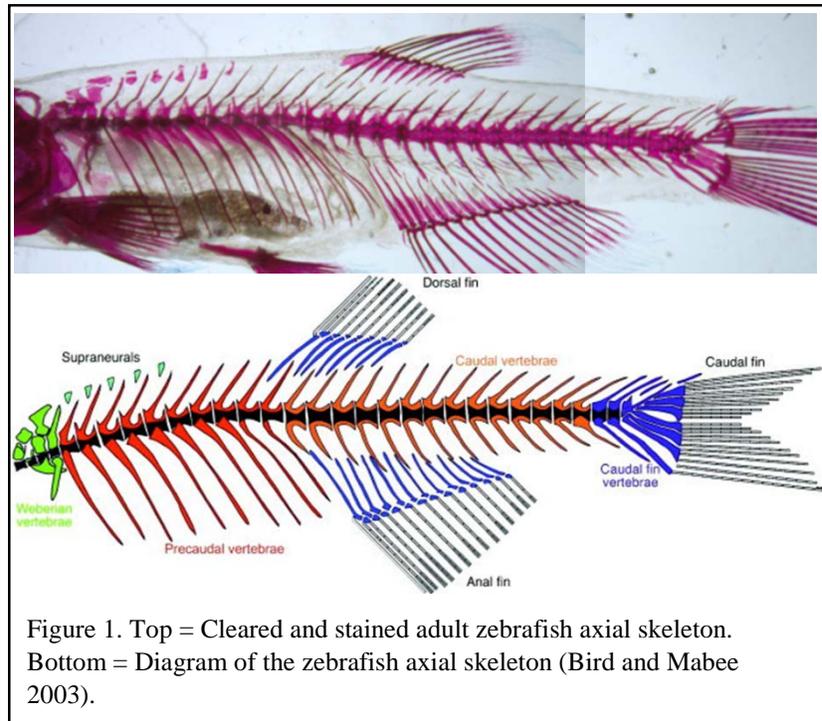
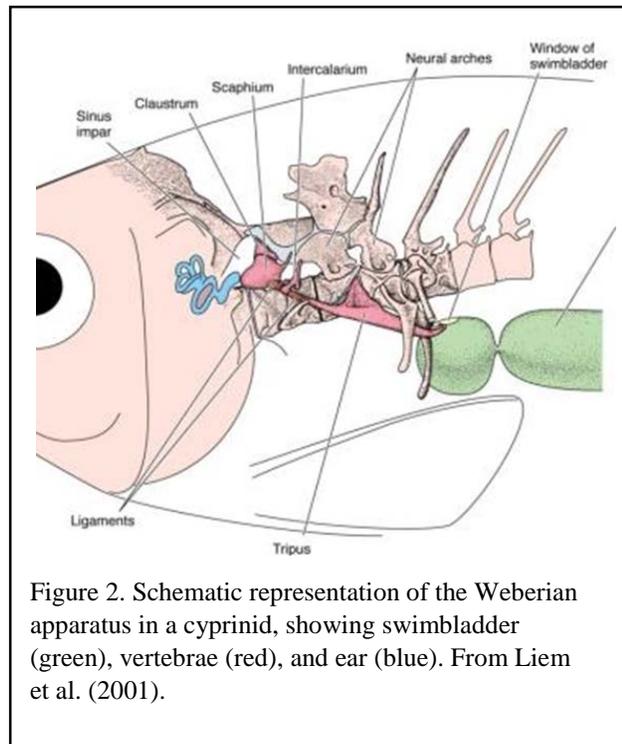


Figure 1. Top = Cleared and stained adult zebrafish axial skeleton. Bottom = Diagram of the zebrafish axial skeleton (Bird and Mabee 2003).

stages of development, when the presomitic mesoderm becomes antero-posteriorly segmented to form somites (Kimmel et al., 1995; Stickney et al., 2000), the precursor tissue for muscle and bone of the axial skeleton. In zebrafish, this process happens very early in development, and is completed at 24 hours post-fertilization (Kimmel et al., 1995). The timing of segmentation is tightly regulated, and alteration of the periodicity can give rise to significant anatomical deformities (Van Eeden et al., 1996). Evolutionarily, changes in timing can lead to lengthening or shortening of the long axis (Ackerly and Ward, 2016). Once the segmented pattern of the somites is complete, cells derived from the somite dissociate and become fated to form the

muscle (myotome), dermis/muscle (dermomyotome), and skeleton (sclerotome) of the post-cranial body (Stickney et al., 2000). The sclerotome then reorganizes as it migrates around the notochord to form the final segmented pattern seen in the adult vertebral column. This reorganization, termed “resegmentation”, is a complex process that relies on the initial segmentation of the somites to establish anterior-posterior polarity along the vertebral column (Morin-Kensicki et al., 2002; Holley 2007). Once complete, the sclerotomal cells from two adjacent somites combine to form a single vertebra (Morin-Kensicki et al., 2002).

Discussion of morphological variation within the vertebral column is often focused on mammalian systems, due to substantial regionalization tied with terrestrial locomotion. However, lesser appreciated variability also occurs in the vertebral column of fishes (Figure 1). One of the most dramatic examples of regionalization in fishes is the Weberian apparatus, a novel hearing adaptation that utilizes modified vertebral elements to



enhance hearing (Figure 2). The Weberian apparatus functions by capturing vibrational input (sound) via the swim bladder, then amplifying and transmitting the sound to the inner ear (Higgs et al., 2003) via the Weberian ossicles, the bony modifications of the vertebrae (Rosen and Greenwood, 1977; Chardon and Vandewalle, 1997). The Weberian apparatus is a functionally-integrated unit (Rosen and Greenwood, 1977), meaning all elements are connected for a specific

function, and the development of each subunit is likely tightly regulated to preserve proper shape and function.

The Weberian apparatus is unique to the Otophysi, an extremely diverse clade that contains over 10,000 species, including minnows, catfishes, characids, and South American electric eels (Berra, 2001). While the origin of the Weberian apparatus has been debated for decades (Rosen and Greenwood, 1977; Chardon and Vandewalle, 1997), no extant otophysan is known to have lost the structure, suggesting strong evolutionary constraint against its loss due to the functional advantage it imparts. Interestingly, significant morphological variation (tied to change in ecology) is present within several otophysan groups, and it has been hypothesized that this variation allows the Weberian apparatus to remain functional in a wide variety of environments (Bird and Hernandez, 2007). The critical functional role of the Weberian apparatus also may have led to constraints during early somite development (Windner et al., 2012).

The fact that the Weberian apparatus has never been lost suggests that the somities that give rise to these vertebrae are buffered from early developmental changes. Such evidence is found in other tissues, such as the dermomyotome and myotome, which are disrupted by the loss of the gene *tbx6* (a mesodermal fate and patterning gene [Holley, 2007]) in posterior somites, but remain normal in the anterior somites that give rise to the Weberian apparatus (Windner et al., 2012). The vertebrae of the Weberian apparatus are normal in zebrafish lacking *tbx6*, while the more posterior vertebrae are dramatically malformed (Bird unpublished data). Other factors, such as temperature, can have a pronounced effect on somite development and skeletal patterning. Previous work showed that temperature variation during development may affect vertebral number in zebrafish (Ackerly and Ward, 2016), which in turn affects the swimming velocity of the fish and thus its survival. However, no study has examined the effect of

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temperature on development of elements of the Weberian apparatus specifically, which could affect a fish's hearing capabilities and in turn affect its survival as well. The purpose of this study is to explore the effect temperature may have on development of elements of the Weberian apparatus in relation to the other regions of the vertebral column.

The goals of this study were three-fold

1. To test rapid whole-mount staining techniques to quickly determine effect of temperature on somite number.
2. To examine the effect of extreme low and extreme high rearing temperature on somite formation.
3. To examine the effect of extreme low and extreme high rearing temperature on skeletal development in the Weberian apparatus.

MATERIALS AND METHODS

Fish Husbandry and Breeding. Wildtype (AB) zebrafish were obtained from Zebrafish International Resource Center (Eugene, OR). Adult zebrafish were maintained at 28.5 +/- 0.5°C on a 12:12 light cycle (standard conditions, following Westerfield, 2000). Adults were fed twice daily, either live newly-hatched brine shrimp (Brine Shrimp Direct, Ogden, UT), or commercial pellets (Pentair Aquatic Ecosystems, Apopka, FL). Larval zebrafish were maintained in incubators at 28°C, and fed live paramecia starting at seven days post fertilization (dpf; Carolina Biological Supply, Burlington, NC), followed by brine shrimp. Once ingestion of brine shrimp was confirmed for all individuals, addition of paramecium was stopped (typically by day 15).

For breeding, adults were placed in breeding chambers (Pentair) the night before collection, with males and females kept separate to prevent overnight mating events. The following morning, barriers were removed and breeding groups were moved to fresh room-

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temperature water within five minutes of first-light, following standard methods (Westerfield, 2000). Breeding groups were monitored for embryo production, and upon completion (no embryos for two consecutive cycles), adults were returned to normal housing and fed.

Embryo collection and sampling. Embryos were collected every 30 minutes to maximize uniformity in developmental timing among individuals within a brood. Once collected, embryos were transferred to a petri dish filled with embryo medium (EM; Westerfield, 2000), then placed in an incubator at 28°C and allowed to develop normally. At ten hours post-fertilization (hpf; end of gastrulation), embryos were screened, and poor quality embryos (dead, unfertilized, or deformed) were removed from the brood. Remaining embryos were split into equal groups and placed in one of three treatments: normal rearing temperature (28°C, Control), extreme low temperature (21°C), or extreme high temperature (33°C), and placed into incubators set for each water temperature. For somite counts and stain testing, larvae were sampled between 24-48 hours of development (based on anatomical development, Kimmel et al., 1995). Larvae were anesthetized using buffered 0.04% Tricaine (MS-222, Ethyl 3-aminobenzoate methanesulfonic acid; Fisher), then fixed in chilled 10% formalin (Fisher) buffered using phosphate buffered saline (PBS) for 24h at 4°C. Larvae used for skeletal analysis were raised in the experimental temperatures between 10-48 hours post-fertilization (during developmental periods when sclerotome in the anterior somites is migrating and differentiating), then transferred back to normal rearing temperature (28°C) and allowed to develop until sampled at juvenile stages.

Rapid staining tests and somite counts. Eight common histological stains were tested for utility as a rapid somite-labeling stain: Methylene Blue, Methyl Green, Nuclear Fast Red, Buffalo Black B, Methyl Red, Eosin Y, Iodine Potassium Iodide (IKI), and Fast Green FCF (Figure 3). For each stain, the following testing regime was utilized. First, larvae were washed in

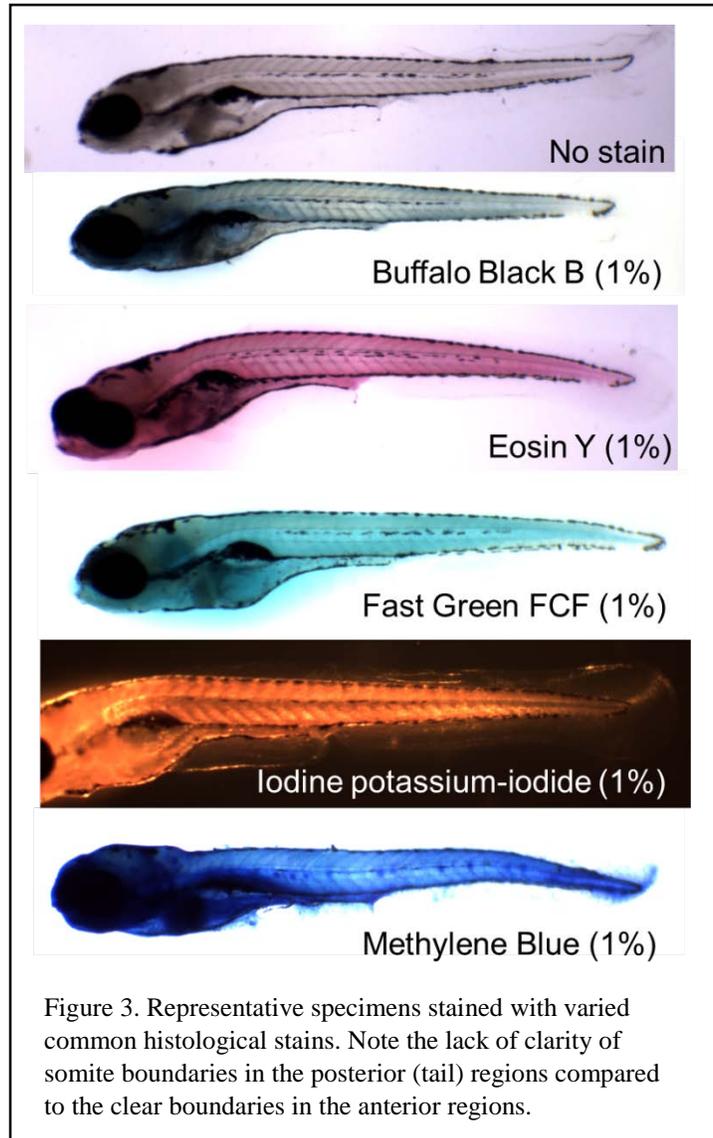
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PBS for 30 minutes to remove residual fixative. Next, larvae were placed in a 1% solution of the particular stain, and remained in the stain for one, three, five, or ten minutes. Larvae were then removed from the stain and placed back into PBS, examined for stain intensity and clarity of somite/myotomal borders, and somites counted (Figure 3).

Somite Counts. Between 18-20 individuals from each of the treatment groups were assessed for somite counts using the rapid staining method (19 larvae from the 28°C rearing group, 20 from the 21°C group, and 18 from the 21°C group). Both total somite number as well as anterior somite number (from somite #1 through the somite at the level of the anal vent) were collected for each individual, in order to determine whether changes in somite number were due to specific regional changes, or random.

The mean number of somites (total and

regional) were compared between experimental groups using ANOVA, followed by Tukey HSD post-hoc tests when ANOVAs were significant (JMP 12, SAS Institute, Inc.). Data on somite number were collected on a Dell Optiplex 960 using a VanGuard 1272ZL dissecting microscope outfitted with a VanGuard IS500 camera. Images were collected with IS Capture.



Immunohistochemistry. To verify the accuracy of the rapid staining method, a second group of larvae were fluorescently labeled using immunohistochemical techniques outlined in Bird et al. (2012). Larvae were double-labeled for Pax7 and MF20 (Developmental Studies Hybridoma Bank). The Pax7 antibody (Mouse IgG1 anti-chicken monoclonal) labels dermomyotome cells, while the MF20 antibody (Mouse IgG2b anti-chicken monoclonal) labels

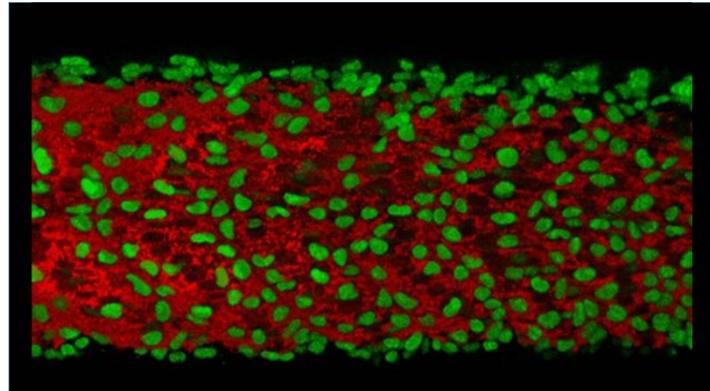


Figure 4. Example of immunohistochemistry (Bird, unpublished). Pax7+ dermomyotome (green) and total muscle myosin (red) reveal the segmentation pattern of the somites.

whole-muscle myosin. Both antibodies reveal somite segmentation patterns even in the smallest somite, allowing for clear and definitive visualization of somite boundaries (Figure 4).

Briefly, the immunohistochemical protocol is as follows. First, embryos stored in methanol are rehydrated to PBS, then rinsed (3 x 1') and washed (3 x 15') in PBS-Tween 20 (Tween 20 is a detergent that aids in tissue permeability, 0.1%). Next, non-specific binding sites are blocked using PBS-Tw-B (B = Bovine Serum Albumin, Sigma, 2%; 1 x 15'), followed by PBS-Tw-B-N (N = Normal Goat Serum, Sigma, 5%, 1 x 15'). Primary antibodies were diluted in PBS-Tw-B-N for a final antibody concentration of 5µg/mL. Embryos remained in primary antibody for 2.5 hours at room temperature while rocking. After incubation, the primary antibodies were removed, and larvae were rinsed (3 x 1') and washed (3 x 15') in PBS-Tween 20. Remaining non-specific binding sites (reopened by washes) were again blocked by subsequent PBS-Tw-B (1 x 15') and PBS-Tw-B-N (1 x 15') treatments. Fluorescently-conjugated secondary antibodies against Pax7 (Goat anti-mouse IgG1-FITC conjugated,

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Southern Biotech) and MF20 (Goat anti-mouse IgG2b-Alexa 546 conjugated, Sigma) were used at 1:800 dilution in PBS-Tw-B-N. Embryos remained in secondary antibodies for 2 hours (in foil), then rinsed (3 x 1') and washed (3 x 15') in PBS-Tween 20, and finally cleared overnight in 50% Glycerol (Fisher) in PBS-Tw. Counts were completed on a Zeiss Axio ScopeA.1 fluorescent microscope.

Skeletal analysis. Juveniles were assessed for defects in the axial skeleton using standard clearing and staining techniques. The procedure followed those outlined by Bird and Mabee (2003), which were modified from original techniques developed for fishes (Dingerkus and Uhler, 1977; Potthoff, 1984). Briefly, fixed specimens were dehydrated to 100% Ethanol, then stained overnight in 0.02% Alcian blue (20% glacial acetic acid in absolute ethanol), which selectively stains cartilage. Next, specimens were rehydrated through a descending ethanol series (95%, 75%, 50%, 25%, Water; 30 minutes each) and transferred into an aqueous saturated sodium borate solution overnight to neutralize any remaining acid. Next, specimens were transferred into 0.5% KOH + H₂O₂ solution for up to 3 hours to remove surface pigmentation, followed by muscle digestion using 1% trypsin solution (30% dH₂O, 70% saturated sodium borate). Once sufficient muscle mass had been cleared and rostral-most vertebrae were becoming visible, specimens were washed to remove the trypsin solution and then stained overnight with a 0.025% Alizarin red solution (in 0.5% KOH) to stain bone. Lastly, specimens were placed in an increasing glycerol series (3:1 KOH: glycerol, 1:1 KOH: glycerol, 3:1 glycerol: H₂O; each step overnight) to finish the clearing process. Specimens were stored in 3:1 glycerol: H₂O for skeletal analysis. Depending on size of the specimen, the entire process can take four days to two weeks; all juveniles for this study were cleared and stained in approximately six days.

RESULTS

Rapid whole-mount staining

Rapid stains exhibited significant variation in efficacy, reliability, and stain duration. For all stains, myotomal boundaries were clearly evident in the anterior half of the body (Figure 3). However, these boundaries are also evident in non-stained specimens under specific lighting conditions, although they were often more pronounced in stained specimens. Posterior myotomal boundaries (posterior to the anal vent, which are notably shorter) were not clearly visible using non-stained specimens or any of the commercial stains except IKI. However even in IKI stained specimens the labeling of

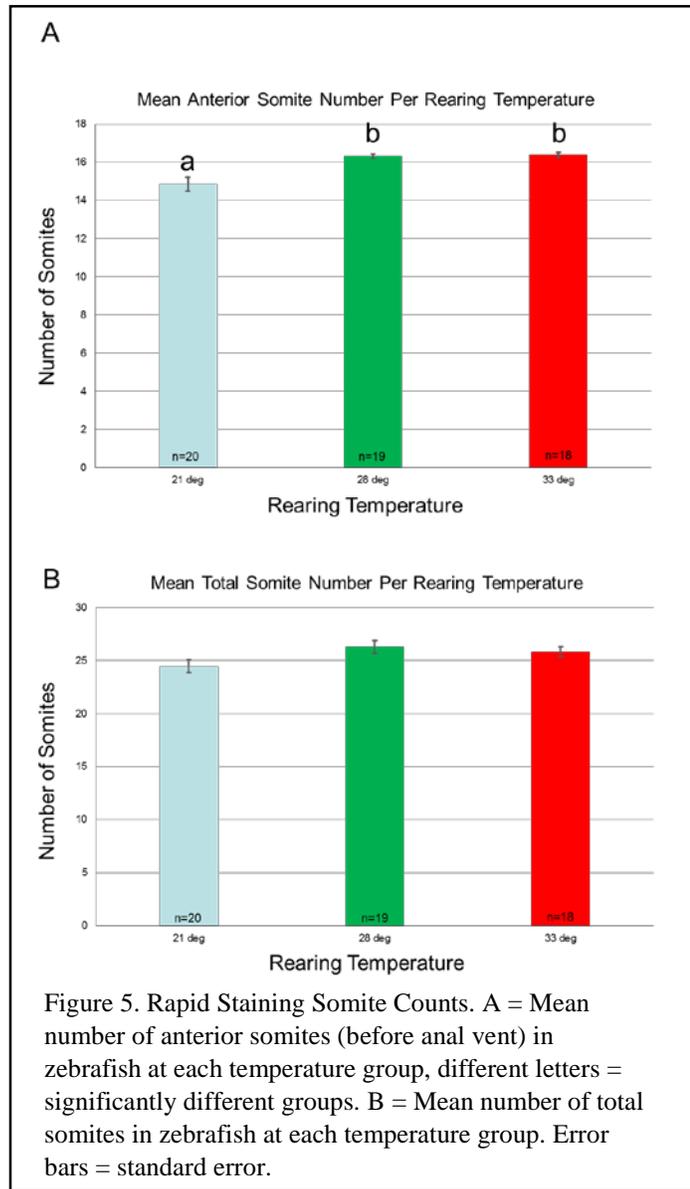


Figure 5. Rapid Staining Somite Counts. A = Mean number of anterior somites (before anal vent) in zebrafish at each temperature group, different letters = significantly different groups. B = Mean number of total somites in zebrafish at each temperature group. Error bars = standard error.

posterior myotomal boundaries was variable (Figure 3). Final qualitative comparison determined that the most effective combination for identifying posterior myotomal boundaries was iodine potassium-iodide (IKI, 1% for 10 minutes) under polarized light (Figure 3). Polarized light takes advantage of the crystalline-like arrangement of the actin proteins within muscle fibers, creating

a “glow” under proper orientations and lighting conditions, which was further enhanced by the IKI. Results of somite counts for the rapid staining technique are based off of counts completed using the IKI/Polarized light protocol. All other stains assessed for rapid somite labeling are not recommended.

Somite Counts

Experimental groups compared using the rapid-stain method revealed no difference among groups for total somite number in larval zebrafish (ANOVA, $F=2.86$, $p<0.07$; Figure 5). However, while not statistically significant, both 21°C and 33°C groups tended to have fewer total somites (21°C ranged from 18-30, 28°C ranged from 25-30, and 33°C ranged from 22-28). In contrast, differences in number of anterior somites was found among treatment groups (ANOVA, $F=13.43$, $p<0.0001$; Figure 5), with fewer somites found in the anterior region of the

21°C treatment group than both the 28°C and 33°C groups. No difference in anterior somite number was found between 28°C and 33°C groups.

Somite counts collected using immunohistochemical methods revealed differences between treatment groups in total

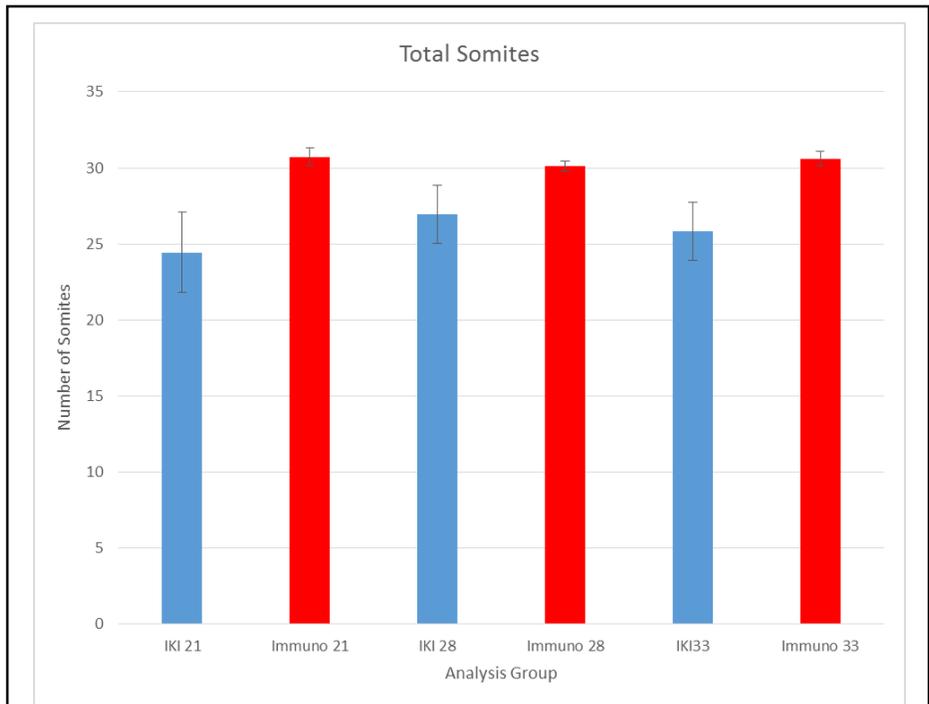


Figure 6. Comparison of total somite numbers in the different analysis methods. Blue=IKI, Red=Immunohistochemistry. Note the counts were consistently higher using immunohistochemistry than IKI, with less variability (see error bars = standard deviation).

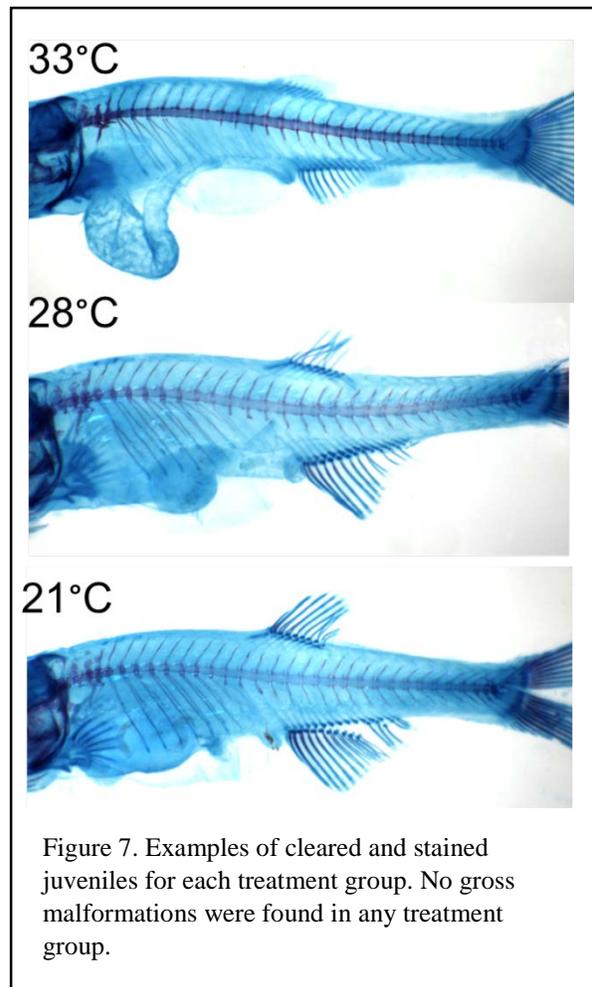
number of somites (ANOVA, $F=6.79$, $p<0.003$). Tukey HSD post-hoc test revealed that both high temperature (mean 30.6 somites, range 30-31) and low temperature (mean 30.7 somites, range 30-32) had a greater number of total somites than control (mean 30.1 somites, range 30-31; Figure 6). Due to the rapid bleaching of specimens, regional somite counts were not able to be obtained.

Comparison of total somite number between analysis methods revealed clear differences in accuracy of somite count between the two methods (Figure 6). Use of immunohistochemical methods produced higher counts for all treatment groups (mean somite counts of 30.7 vs. 24.5 in 21°C group, 30.1 vs. 26.8 in 28°C group, and 30.6 vs. 25.8 in 33°C group), and were more consistent within groups (note error bars in Figure 6). The results found in using

immunohistochemistry also better coincide with somite numbers found in previous developmental descriptions (30-34 somites; Kimmel et al. 1995, Stickney et al. 2000).

Skeletal Development

For each treatment group, larval survival to adulthood was low (4.4% for 21°C, 5.6% for 28°C, and 4.4% for 33°C). Within each group, anatomical structures of the vertebral column were compared to the morphological description of wildtype zebrafish axial skeleton development and intraspecific variation documented in Bird and Mabee (2003). This included centra, neural arches and spines, hemal



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arches and spines, parapophyses and ribs, elements of the Weberian apparatus (scaphium, claustrum, intercalarium, tripus, os suspensorium), and hypurals of the caudal fin. No distinct skeletal deformities were found in any region of the vertebral column (including the Weberian apparatus) in all treatment groups (Figure 7). In addition, since vertebral number is directly associated with somite number (Morin-Kensicki et al., 2002; Stickney et al., 2000), vertebral counts were collected for each region and compared via ANOVA. No difference was found between treatment groups for any vertebral region (see Table 1 for statistics). The number of Weberian vertebrae and caudal fin vertebrae were invariant for all groups (4 and 3 vertebrae, respectively). Some variation was found among groups for all other vertebral regions, but did not reach the level of statistical significance among treatment groups.

Mean Vertebral Number				
	Range	F	d.f.	P
Temperature by Total Vertebrae	31-32	2.0234	2, 10	0.1829
Temperature by Weberian Vertebrae	4	Invariant		
Temperature by Thoracic Vertebrae	8-9	1.1538	2, 10	0.3541
Temperature by Transitional Vertebrae	2-3	1.1538	2, 10	0.3541
Temperature by Hemal Vertebrae	14-15	0.4945	2, 10	0.624
Temperature by Caudal Fin	3	Invariant		

Table 1. ANOVA analysis on data collected by counting the total vertebrae in each of the listed skeletal regions. Though there was variability within the middle groups of vertebrae, ANOVA testing revealed no significant differences in total vertebrae or regional vertebrae between temperature groups. Temperature by Weberian vertebrae showed no variability across temperature groups and was not analyzed using ANOVA.

DISCUSSION

Efficacy of Rapid Stains

Results for the histological stains have revealed limited utility for the identification of somite/myotomal borders in whole-mount specimens. Myotomal boundaries were not clearly visible in posterior vertebral regions using any of the stains we tested except for IKI. Using IKI, all myotomal boundaries were visible in some but not all specimens. While IKI was the most effective, the presence of substantial variability in posterior somite identification resulted in low confidence in the accuracy of IKI somite counts. The predicted low accuracy was confirmed after comparison to mean somite number in specimens stained using immunohistochemistry, which was significantly higher than IKI stained specimens in all treatment groups. This suggests that while qualitatively better than the other tested stains, significant numbers of small somites (with smaller borders) were still missed using IKI. As a result, none of the tested rapid stains should be considered useful or reliable for assessing somite number in zebrafish embryos. While more time consuming and labor intensive, the accuracy, repeatability, and reliability of the immunohistochemical method is far superior and should be used.

Temperature and somitogenesis

Overall, the data collected via immunohistochemistry provide clear indication that temperature does affect overall somite number. Both high and low temperature treatments appear to perturb proper temporal regulation of somitogenesis (Holley, 2007), as both showed a statistical increase in the number of somites compared to control. While overall somite number from IKI-stained specimens is suspect, the anterior counts are likely accurate due to the size and clarity of borders, even in whitelight analysis. The variability in mean anterior somite number in specimens raised at low temperatures compared to other treatment groups may also suggest a

potential role of shifting *hox* gene boundaries between anterior and posterior regions (Morin-Kensicki et al., 2002) in addition to a change in rate of somitogenesis (Kimmel et al., 1995).

Effects of Temperature on Skeletal Development

Different rearing temperatures did not cause significant variability in vertebral number. Mean number of vertebrae was not different between treatment groups in any vertebral region; however, some interesting trends were identified. The zebrafish raised in 21°C and 33°C had a slight increase in the number of total vertebrae and hemal vertebrae. The zebrafish raised in 33°C had a slight increase in thoracic vertebrae, and those raised in 21°C had a slight increase in transitional. There was no variability found among treatment groups in the number of Weberian vertebrae or the caudal fin vertebrae, suggesting that both of these regions are buffered against any developmental changes due to temperature. The limited survival of the larvae (13 out of 270) may have hindered the detection of variation within vertebral regions, and a more robust analysis with a larger number of fish within each treatment group is recommended to confirm any of the noted trends. The time the fish were left in their respective temperature incubators (28 hpf for 33°C, 24 hpf for 21°C) may not have been long enough to affect the migration or differentiation of the sclerotome to the degree that would result in the development of any noticeable effects within different vertebral regions.

In addition to the lack of variability in vertebral number, morphogenesis of the vertebral elements also proceeded normally regardless of temperature in all vertebral regions. This finding suggests that variability in rearing temperature does not play a significant role in the morphogenesis of vertebral elements. However, as with the regional vertebral number, the limited survival of the larvae paired with the short duration in treatment temperature may have hindered the detection of variation in morphogenesis of vertebral elements.

Temperature in the context of somitogenesis and vertebral development

Previous studies on the effect of temperature on vertebral number in zebrafish show both similarities and differences to the results of this study. The study conducted by Ackerly and Ward (2016) showed that temperature had no significant effect on precaudal (hemal in this study) or total vertebral number. While this study also found no difference in overall number of somites, changes in other vertebral traits (e.g. caudal vertebrae number) were found. Individuals reared at their lowest temperature (24.5°C) had the highest mean number of caudal vertebrae (18.6), while those at their intermediate temperatures (26.5°C and 28.5°C) had the lowest average caudal vertebral number. Fish raised at the highest temperature (30.5°C) had the second highest average (18.1). Though not significant, their study also found that individuals raised at the highest temperature (30.5°C) had a slightly higher average vertebral number than intermediate temperatures (32.6 for 30.5°C, 32.1 for 26.5°C, 32.2 for 28.5°C), while those raised at the lowest temperatures (24.5°C) had the highest average total vertebral number (32.9).

Both this study and Ackerly and Ward (2016) found no effect of temperature on the total number of vertebrae. However, Ackerly and Ward (2016) found significant variability within the vertebral regions, specifically the number of caudal vertebrae, while this study did not. The differences in the studies could be due to a difference in experimental methods. In Ackerly and Ward (2016), fish were kept at temperatures of 24.5°C, 26.5°C, 28.5°C, and 30.5°C. In this study, the more extreme temperatures (21°C and 33°C) may have hindered survival, causing our final test subject group to be smaller. Furthermore, embryos in Ackerly and Ward (2016) were placed directly into experimental temperature tanks, except the 24.5 embryos, which were hatched in an incubator for 2 weeks before being placed in 24.5°C tank. This was done to improve survival of that temperature group. They kept the fish in their respective temperature

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incubators for six weeks after hatching before moving them to 28.5°C incubator. Differences in this study were that the fish were not placed in the experimental temperatures until 10 hpf, and they were only kept in experimental temperatures for 28 hpf (33°C) and 24 hpf (21°C). Not allowing the fish to go immediately into experimental temperatures may have kept them from developing any variation during the beginning stages of development. Furthermore, only allowing the fish to be reared in the variant temperatures until 28 hpf and 24 hpf may not have allowed enough time for them to develop variation in the experimental temperatures.

The Weberian apparatus is a functionally-integrated unit (Higgs et al., 2003), and the development of each subunit is tightly regulated to preserve proper shape and function. The development of each subunit is predicted to be buffered from developmental perturbations that affect their ancestral units due to the strong functional constrain on the Weberian apparatus (e.g., developmental irregularities that result in significant morphological defects in non-Weberian vertebrae will not cause defects in the Weberian ossicles). An example of buffering is the morphological manifestation of the loss of the *tbx6* gene (*fused somites*, *fs*) in the axial skeleton. In *fs*, segmentation of the mesoderm is lost, causing widespread defects in the patterning of the presomitic mesoderm (Windner et al., 2012). This patterning breakdown results in massive defects in the axial skeleton in adults (van Eeden et al., 1996); however, in these mutants the elements of the Weberian apparatus are unaffected (Bird unpublished data), suggesting the somites that give rise to the Weberian apparatus are buffered against the loss of *tbx6*.

Similarly, this study shows that the Weberian apparatus remains unaffected to further developmental perturbations, e.g. temperature. Though this study didn't find significant variation among other vertebrae, it also found no variation among Weberian vertebrae or other elements of

the Weberian apparatus. This finding suggests that Weberian apparatus is also buffered against developmental changes caused by changes in rearing temperature.

CONCLUSION

Three goals of this study were addressed. First, rapid whole-mount staining techniques were conducted. Results showed that none of the examined stains were useful to quickly determine effect of temperature on somite number. Second, the effect of extreme low and extreme high rearing temperature on somite formation was examined. Results showed an effect on total somite number (higher somite number in both low and high temperature compared to control in specimens compared using immunohistochemical methods), and a likely effect on number of anterior somites in the low temperature group. Third, the effect of extreme low and extreme high rearing temperature on skeletal development in the Weberian apparatus was examined. Results showed no effect on number of Weberian vertebrae and no defects in the morphology of any element within the Weberian apparatus, suggesting the development of the Weberian apparatus is buffered from developmental changes produced by variation in rearing temperature.

FUTURE DIRECTIONS

Because there were no statistically significant differences found in skeletal development between temperature groups, further experiments can be conducted to verify results and determine if there are other environmental conditions that will result in developmental perturbations within the Weberian apparatus. These projects consist of a similar experiment using different rearing methods to encourage a higher survival rate, and thus have higher specimen numbers to verify results (e.g., start with higher specimen numbers). The same experiment can be run increasing the time the embryos remain in experimental temperatures to

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allow more time for variations in development to occur. Future projects may expand beyond temperature to other potential modulators of somitogenesis and sclerotome differentiation and patterning, including exposing the fish to different chemicals in their rearing environment.

REFERENCES

- Ackerly KL, Ward AB. 2016. How temperature-induced variation in musculoskeletal anatomy affects escape performance and survival of zebrafish (*Danio rerio*). *Journal of Experimental Zoology A: Ecological Genetics and Physiology* 325:25-40.
- Berra T.M. 2001. Freshwater Fish Distribution. Academic Press, San Diego. 604 p.
- Bird N.C. and Hernandez L.P. 2007. Morphological variation in the Weberian apparatus of Cypriniformes. *Journal of Morphology* 268:739-757.
- Bird N.C. and Mabee P.M. 2003. Developmental morphology of the axial skeleton of the zebrafish, *Danio rerio* (Ostariophysi: Cyprinidae). *Developmental Dynamics* 228:337-357.
- Bird, N.C., Windner, S.E., Devoto, S.H. 2012. Immunocytochemistry to Study Myogenesis in Zebrafish. In DiMario, J. X. (ed.), Myogenesis: Methods and Protocols. *Methods in Molecular Biology* 798:153-169.
- Chardon M. and Vandewalle P. 1997. Evolutionary trends and possible origin of the Weberian apparatus. *Netherlands Journal of Zoology* 47:383-403.
- Dingerkus G. and Uhler D. 1977. Enzyme clearing of Alcian blue stained whole small vertebrates for demonstration of cartilage. *Stain Technology* 52:229-232.
- Higgs D.M., Rollo A.K., Souza M.J., and Popper A.N. 2003. Development of form and function in peripheral auditory structures of the zebrafish (*Danio rerio*). *Journal of the Acoustical Society of America* 113:1145-1154.

REARING TEMPERATURE AND DEVELOPMENT IN ZEBRAFISH

- Holley S.A. 2007. The genetics and embryology of zebrafish metamerism. *Developmental Dynamics* 236:1422-1449.
- Kimmel C.B., Ballard W.W., Kimmel S.R., Ullmann B., and Schilling T.F. 1995. Stages of embryonic development of the zebrafish. *Developmental Dynamics* 203:253-310.
- Liem K.F., Bemis W.E., Walker W.F. Jr., and Grande L. 2001. Functional anatomy of the vertebrates: an evolutionary perspective. 3rd ed., Harcourt College Publishers, Fort Worth, TX.
- Morin-Kensicki E.M., Melancon E., and Eisen J.S. 2002. Segmental relationship between somites and vertebral column in zebrafish. *Development* 129:3851-3860.
- Potthoff T. 1984. Clearing and staining techniques. In: H.G. Moser, ed. *Ontogeny and systematics of fishes*. Allen Press, Lawrence, KS. pp. 35-37.
- Rosen D.E. and Patterson P.H. 1970. Origin of the Weberian apparatus and the relationships of the ostariophysan and gonorynchiform fishes. *American Museum Novitates* 2428:1-25.
- Stickney H.L., Barresi M.J.F., and Devoto S.H. 2000. Somite development in zebrafish. *Developmental Dynamics* 219:287-303.
- van Eeden F.J., Granato M., Schach U., Brand M., Furutani-Seiki M., Haffter P., Hammerschmidt M., Heisenberg C.P., Jiang Y.J., Kane D. A., Kelsh R.N., Mullins M.C., Odenthal J., Warga R.M., Allende M.L., Weinberg E.S., Nusslein-Volhard C. 1996. Mutations affecting somite formation and patterning in the zebrafish, *Danio rerio*. *Development* 123:153-164.

REARING TEMPERATURE AND DEVELOPMENT IN ZEBRAFISH

Westerfield M. 2000. The zebrafish book: A guide for the laboratory use of zebrafish (*Danio rerio*). 4th ed., Univ. of Oregon Press, Eugene, OR.

Windner S.E., Bird N.C., Doris R.A., and Devoto S.H. 2012. *Fss/Tbx6* is required for central dermomyotome cell fate in zebrafish. *Biology Open* 1: 806-814.

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