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Testing for modularity in the axial skeleton of fishes

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TESTING FOR MODULARITY IN THE AXIAL SKELETON OF FISHES

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ABSTRACT

The Weberian apparatus is a novel hearing adaptation of otophysan fishes (including such fishes as minnows, loaches, catfishes, characids, and South American electric eels) that allows for dramatically increased hearing capability and sensitivity. The strong functional advantage otophysans gain via the Weberian apparatus has likely created a new modular unit (set of structures that develop, evolve, and function in concert). To determine if components of the Weberian apparatus are integrated into a new developmental module, the timing and sequence of development was collected for specific anatomical structures related to the Weberian apparatus to determine developmental sequence. Patterns of development within species revealed a shift in developmental timing for elements of the Weberian apparatus in zebrafish, relative to the sequence position of the homologous structures in a cichlid, which does not have a Weberian apparatus. These results support a hypothesis for the elements of the Weberian apparatus representing a unique developmental module.

INTRODUCTION

Modularity and Integration

Modules are parts of a network that form a hierarchy of structures within an organism, and can be classified in several different contexts (Klingenberg, 2008). In vertebrate systems, a set of structural elements can be grouped together by a common function (functional module), by position within the organism (anatomical module), by common developmental timing and order (developmental module), or by common gene expression and/or regulation (genetic module). While often linked, groups of elements may be classified as one type of module, but not others. A common feature of modules is that elements within the module tend to exhibit correlated patterns of evolution within the module, but evolve independently from elements outside the module.

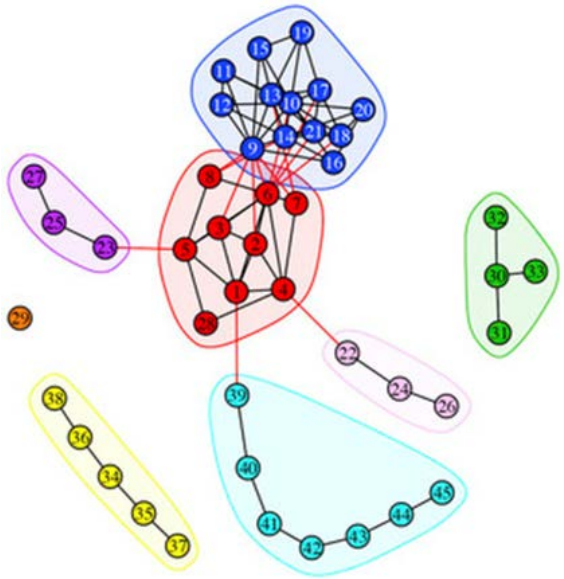

Figure 1. Diagram representing different modules (by color) and the interactions within and among them. From Esteve-Altava et al. 2015.

In addition to identifying the independence of individual modules, it is also important to identify the ways that modules may interact with one another, also known as modular integration. Each module exists with its own level of independence and integration within the organism as a whole. For example, the network shown in Figure 1 shows several different modules in varying states of independence and integration. Some modules are completely independent, with internal elements only interacting with other elements within the module (yellow, green). Other modules show very limited

interaction with other modules (purple, teal, and pink). Still other modules show extensive integration with other modules (red, blue).

There are several different methods to test for the presence of a module. For example, a proposed functional module can be tested by removing elements individually and determining if the function has been compromised. For developmental modules, data on timing and sequence can be collected to determine if there is a correlation between the process of development among structures. If the structures thought to compose a new developmental module are found to develop individually at various times within a set time frame, it is not likely that a new module has formed. However, if it is observed that the structures in question are forming at relatively the same time, then the data would suggest the elements may compose a developmental module. In fishes, the vertebral column is categorized into different modules based on function and anatomy, and therefore may also be composed of different developmental modules.

The Vertebral Column as a Model for Modularity

The vertebral column is the namesake of all vertebrate species, and has been a focus of in depth scientific study for nearly a century (Goodrich, 1930). The vertebral column forms a functional module as the main support structure within the vertebrate trunk, and forms the

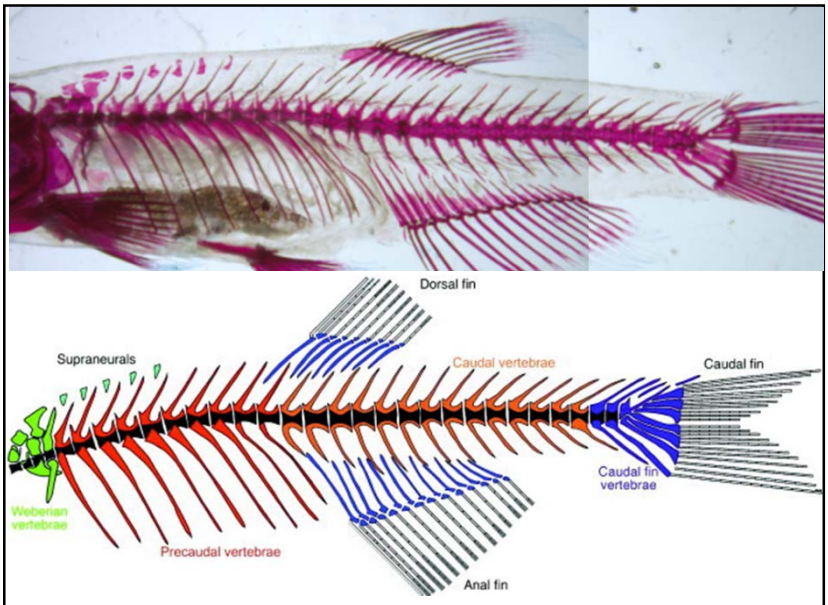

Figure 2. Top = Cleared and stained adult zebrafish axial skeleton. Bottom = Diagram of the zebrafish axial skeleton (Bird and Mabee 2003), showing potential modules in different colors.

longitudinal axis of the fish's body. It serves several functions, including providing surfaces for muscle attachment, protection of the spinal cord, and providing protection of and giving support to the internal organs. The vertebral column can be considered a module within the axial skeleton, with limited integration with the other modules of the axial skeleton, such as the median fins (Figure 2). Within the vertebral column, several submodules can be seen regionally, such as the precaudal and caudal vertebrae shown in Figure 2 (bottom), each having potentially unique functional and developmental roles.

A broad search of the literature shows that much of the interest in the development and evolution of the vertebral column has focused on terrestrial vertebrates, which display dramatic regionalization and specializations correlated to numerous different functional roles (Liem et al. 2001). However, surprising modifications can also be found in the vertebral column of more ancestral vertebrates, such as in bony fishes (Bird and Mabee 2003, Figure 2). One such modification is the Weberian apparatus (Bird and Hernandez 2007, Figure 3), a novel hearing adaptation of otophysan fishes, a large group of bony fishes that include minnows, loaches, catfishes, characids, and South American electric eels. The Weberian apparatus has been implicated as a key innovation in the explosive radiation of otophysan fishes (Early Triassic; Nakatani et al. 2011), one of the most speciose groups of vertebrates (10,000+ species; Berra 2001).

Broadly, the Weberian apparatus is a relay system and sound amplifier, collecting near- and far-field sound inputs via the swim bladder, then redirecting and amplifying the vibration using the Weberian ossicles (modifications of the skeletal elements of the first four vertebrae). Sound input is then transmitted forward to the inner ear (Figure 3). The apparatus allows for dramatically increased hearing capability across a much wider bandwidth than non-otophysan fishes, and detection requires a reduced magnitude threshold (Schellart and Popper 1992, Higgs et al. 2003). This adaptation is analogous to the middle ear of mammals, which also uses a system of modified bones to relay and amplify sound. The Weberian apparatus is composed of several different adaptations acting in concert (skeletal modifications in the vertebral column, and novel changes in ligaments, ligamentous attachments, and tissues of the swim bladder and inner ear). All of these varied adaptations have been observed to work together as a single functional module (Ladich and Wysocki, 2003), but it has not been determined whether individual elements of the Weberian apparatus develop in concert with each other, or develop independently.

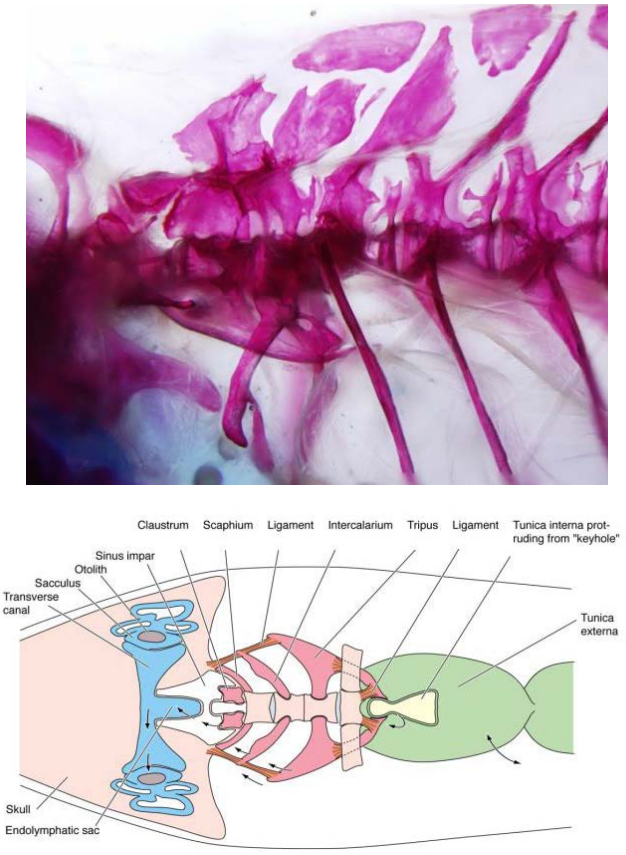

Figure 3. Top = close-up image of the Weberian apparatus in a cleared and stained adult zebrafish. Bottom = diagram of the Weberian apparatus (dorsal view), showing the relationships and connections between the ear (blue), vertebral column (red), and swim bladder (green). Bottom image from Liem et al. 2001.

Hypothesis and Testing

The structures of the Weberian apparatus, which includes modified portions of the vertebrae, swim bladder, and inner ear, form a well-defined and distinct functional unit (Ladich et al. 2003) as well as an integrated anatomical unit (Bird and Mabee 2003). Previous studies of variability within cypriniform species also suggest that the unit evolves as a single unit (Bird and Hernandez 2007), suggesting the formation of a unique developmental and evolutionary module. To determine whether the Weberian apparatus is a developmental module, the sequence of development of elements of the Weberian apparatus in the zebrafish, *Danio rerio* (herein referred to as *Danio*), was assembled and compared to the sequence of development of the homologous structures in a cichlid, *Tramitichromis* sp. (herein referred to as *Tramitichromis*), which does not possess a Weberian apparatus.

Shifts in developmental timing between species can be evidence for sequence heterochrony (evolutionary change in developmental timing in a descendant relative to an ancestor; Smith 2002), and correlated developmental shifts in functionally correlated structures is evidence for an integrated developmental module. Development within a generalist teleost skull follows a typical sequence (Cubbage and Mabee 1996, Mabee et al. 2000): functional adaptations within the skull can cause evolutionary shifts in developmental timing, such that structures shift earlier or later in development depending on the nature of the functional demand, such as feeding. Those structures not involved generally remain static in sequence, allowing comparisons among species to isolate structures that have shifted in evolutionary timing. The same principle can be expanded to other regions of the vertebrate skeleton. Shifts in timing between species can be evidence for sequence heterochrony (evolutionary change in developmental timing in a descendant relative to an ancestor), and correlated developmental

shifts in functionally correlated structures is evidence for an integrated developmental module. By comparing species with and without a Weberian apparatus, I tested the following hypotheses:

Hypothesis 1: *The subunits of the Weberian apparatus have shifted in developmental timing away from their ancestral units and are temporally linked to form a new integrated developmental module.*

Hypothesis 2: The subunits of the Weberian apparatus are decoupled from their ancestral units, but are not temporally linked and have not been captured into a new integrated developmental module.

Hypothesis 3: The subunits of the Weberian apparatus are not decoupled from their ancestral units.

If elements of the Weberian apparatus have shifted to become a new integrated modular unit, it is expected that these elements will develop at similar times ontogenetically, most likely in order to gain proper functionality as early as possible. In non-Weberian species, the expectation is that the structures homologous to the Weberian apparatus will not develop at the same time ontogenetically, as they all have disparate functions, and their developmental timing would be grouped with other elements of their ancestral function. The first two hypotheses predict two scenarios that detect sequence heterochronies. The first represents a fully integrated module, while the second represents a "module in the making", whereby the structures have become dissociated from their ancestral anatomical units, but the new module is not detected developmentally. If elements of the Weberian apparatus show the same sequence of development as in the non-Weberian species, hypotheses 1 and 2 would be rejected, and hypothesis 3 supported.

MATERIALS AND METHODS

Fish Husbandry and Breeding. Adult wildtype (AB) zebrafish were obtained from the Zebrafish International Resource Center (Eugene, OR) in May 2015. 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 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 (Carolina Biological Supply, Burlington, NC) starting at seven days post fertilization (dpf), followed by brine shrimp. Once individuals were confirmed to be eating brine shrimp, addition of paramecia was halted (usually 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-temperature water within five minutes of first-light, following standard methods (Westerfield 2000).

Embryo collection and sampling. An ontogenetic series of the cichlid *Tramitichromis*, previously fixed and stored in 10% buffered formalin, was kindly provided by Dr. Jacqueline F. Webb (University of Rhode Island); these specimens ranged in size from 4–22 mm Standard Length (SL). Size/length is the standard reporting measure of development for fish, rather than age, as several factors (e.g., temperature, overcrowding, water chemistry, etc.) can substantially affect growth rates. An ontogenetic series of *Danio* (zebrafish) was raised and fixed at various stages of development to match the cichlid series. Embryos were collected every 30 minutes to maximize uniformity in developmental timing among individuals. 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. Fish were anesthetized using buffered 0.04%

Tricaine (MS-222; Fisher Scientific, Cat# AC118000500), then fixed in chilled 10% formalin (Fisher) buffered using phosphate buffered saline (PBS) for 24h at 4°C. The zebrafish ontogenetic series ranged in size from 3.6 mm Notochord Length (NL) to 20.8 mm SL.

Clearing and Staining. The protocol for clearing and staining follows that described in clearing and staining techniques 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 a 0.02% Alcian blue solution (20% glacial acetic acid in absolute ethanol), which stains cartilage. Next, specimens were rehydrated through a descending ethanol series (95%, 75%, 50%, 25%, Water; 30 min. each) and transferred into an aqueous saturated sodium borate solution overnight to neutralize any remaining acid. Next, specimens were transferred into 0.5% potassium hydroxide (KOH) with five drops of 35% H_20_2 added, then incubated for 60-90 min. under direct light to remove surface pigmentation, followed by muscle digestion using a 1% trypsin solution in 7:3 dH_20: saturated sodium borate. Once sufficiently clear and skeletal elements were visible and discernable, specimens were placed in a solution of 0.025% Alizarin red in 0.5% aqueous potassium hydroxide overnight to stain bone. Then, specimens were placed in an increasing glycerol series (3:1 0.5% KOH: glycerol, 1:1 KOH: glycerol, 3:1 glycerol: H_2O; each step overnight) to finish the clearing process. Specimens were kept in 3:1 glycerol: dH_2O while working with them. Depending on the size of the specimen, the entire process can take four days to two weeks.

Developmental Data Collection. Data taken from specific structures (Table 1, Figure 4) on developmental timing and progress of development (whether structures, if present, have chondrified or ossified) 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 (Figure 4). For each species, structures were marked as present or absent, and if present, whether it was cartilage or bone. This provides a relative sequence of when structures develop relative in time to other structures. Species were analyzed by relative sequence of development of Weberian apparatus structures against control structures, and then sequences were compared between species to identify shifts in development.

Table 1. List of structures examined.

	Tramitichromis sp. (cichlid)	*Danio rerio* (zebrafish)		*Tramitichromis* sp. (cichlid)	*Danio rerio* (zebrafish)
VERTEBRAL	Centrum 1		MEDIAN FINS	Dorsal Fin Rays	
	Centrum 2			Dorsal Fin Radials	
	Centrum 3			Anal Fin Rays	
	Centrum 4			Anal Fin Radials	
	Centrum 5			Hypural 1	
	Centrum 6			Parhypural	
	Neural Arch and Spine 1	Scaphium		Caudal Fin Ray	
		Claustrum		Penultimate Centrum	
	Neural Arch and Spine 2	Intercalarium		Urostyle	
	Neural Arch and Spine 3	Neural Arch 3	SKULL	Premaxilla	
	Neural Arch and Spine 4			Maxilla	
	Neural Arch and Spine 5			Dentary	
	Neural Arch and Spine 6			Opercle	
	Lateral Process 1			Preopercle	
	Lateral Process 2			Prootic	
	Parapophysis 3	Tripus (body)		Basioccipital	
		Tripus (processes)		Exoccipital	
	Parapophysis 4			Utricular Otolith	
	Parapophysis 5			Saccular Otolith	
	Parapophysis 6			Lagenar Otolith	
	Rib 3		ORGAN	Swim Bladder	
	Rib 4	Rib 4			
		Os suspensorium			
	Rib 5				
	Rib 6				

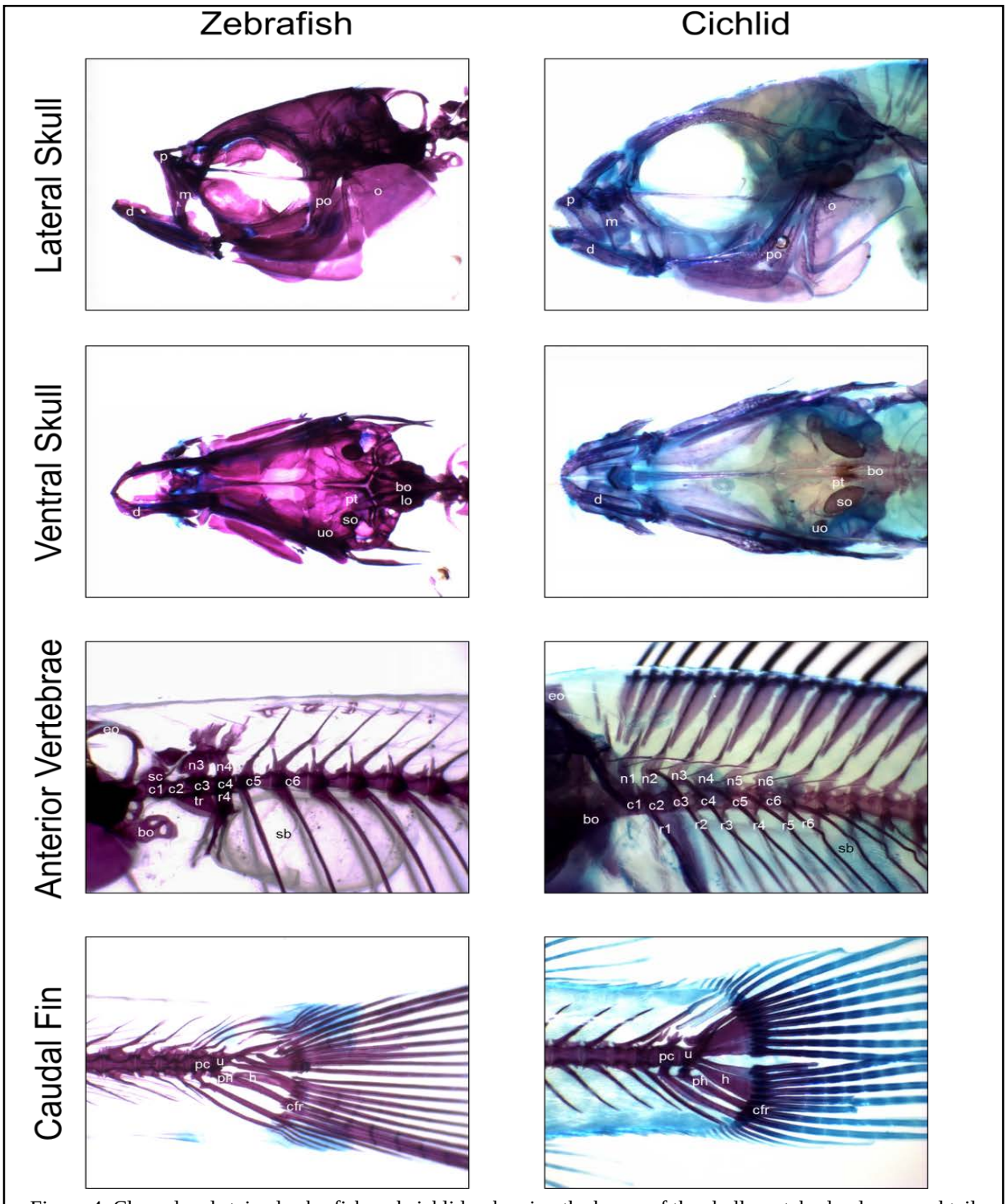

Figure 4. Cleared and stained zebrafish and cichlids, showing the bones of the skull, vertebral column, and tail used in the developmental sequence analysis. bo=basioccipital, c=centrum, cfr=caudal fin ray, d=dentary, eo=exoccipital, h=hypural 1, lo=lagenar otolith, m=maxilla, n=neural arch, o=opercle, p=premaxilla, pc=penultimate centrum, ph=parhypural, po=preopercle, pt=prootic, r=rib, so=saccular otolith, u=urostyle, uo=utricular otolith.

RESULTS

Developmental sequence in *Tramitichromis*

Most elements in all regions (skull, fins, and vertebrae) began developing early in the ontogeny of *Tramitichromis*. Structures became visible between 6–8 mm SL (Figure 5). Interestingly, the sensory-related elements of the ear (otoliths) were delayed in development compared to the skeletal elements of the otic region (i.e., basioccipital, prootic, exoccipital) (Figure 5, blue bars at top and bottom of the chart). Vertebral elements (Figure 5, red) were mixed throughout the developmental sequence, with most being formed early in development. The swim bladder (Figure 5, green) was the last element to form.

With reference to the elements homologous to the Weberian apparatus, no common developmental timing was found in *Tramitichromis*. The receptor element (swim bladder) and processing elements (otoliths) developed late, and significantly delayed compared to the transmissive elements (vertebrae). Overall for all elements, an early wave of development can be seen throughout the body, with development in the head and vertebral column happening simultaneously. A lag in development was then seen between 8–12 mm SL, followed by development of the otoliths. This was followed by another lag until 17 mm SL, when the swim bladder inflated and became visible. While it is possible that the tissue of the swim bladder may be present before inflation, its inflation is critical to both function in buoyancy regulation and hearing in otophysans. Therefore, in the context of this study, it is not considered “present” until inflation.

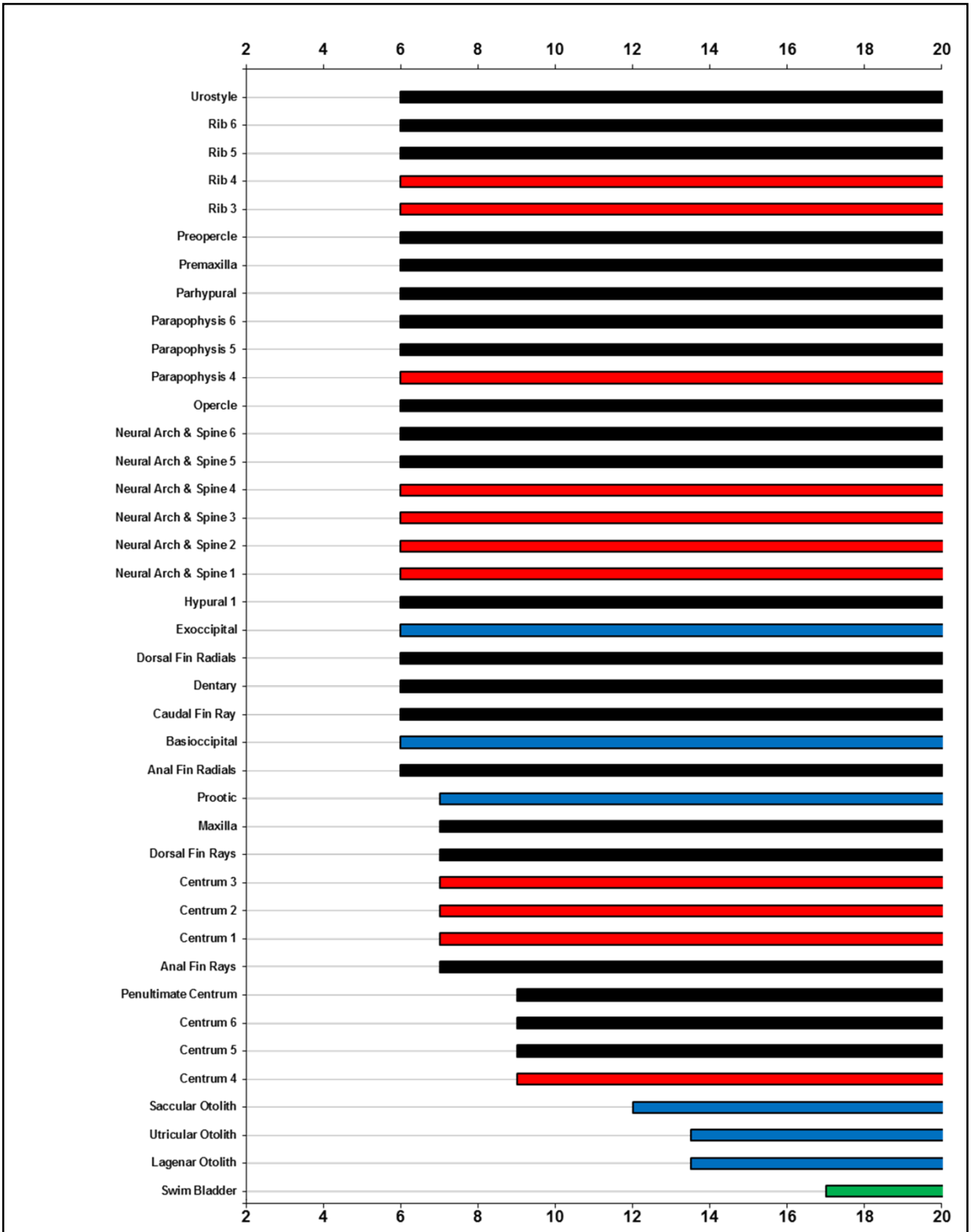

Figure 5. Sequence of development in *Tramitichromis*, showing the smallest size at which individual elements are always present, from earliest (top), to latest (bottom). Numbered axis represents size (mm SL). Colored bars represent elements homologous to parts of the Weberian apparatus (blue = ear, red = vertebral, green = swim bladder). Black bars = reference structures.

Developmental sequence in *Danio*

Similar to *Tramitichromis*, elements in all regions (skull, vertebrae, fins) started developing very early in the ontogeny of zebrafish (Figure 6). However, unlike *Tramitichromis*, all of the elements began their development much earlier, between the lengths of 3.6 mm NL to 6.8 mm SL. No marked lags were found in the developmental sequence, with all elements present by 8.0 mm SL. Within the Weberian apparatus, elements of the ear (Figure 6, blue) develop much closer in ontogeny to each other, as do most elements of the vertebral column (Figure 6, red). The swim bladder (Figure 6, green) remained one of the last elements to form in zebrafish; however, it developed at the same time as vertebral elements of the Weberian apparatus, and was also synchronized with structures of the ear. Interestingly, the saccular otolith, which receives input from the Weberian ossicles, is delayed compared to other otoliths, and is nested in the developmental sequence with the vertebral elements and the swim bladder. Overall, development appears to occur much earlier and more rapidly in the zebrafish.

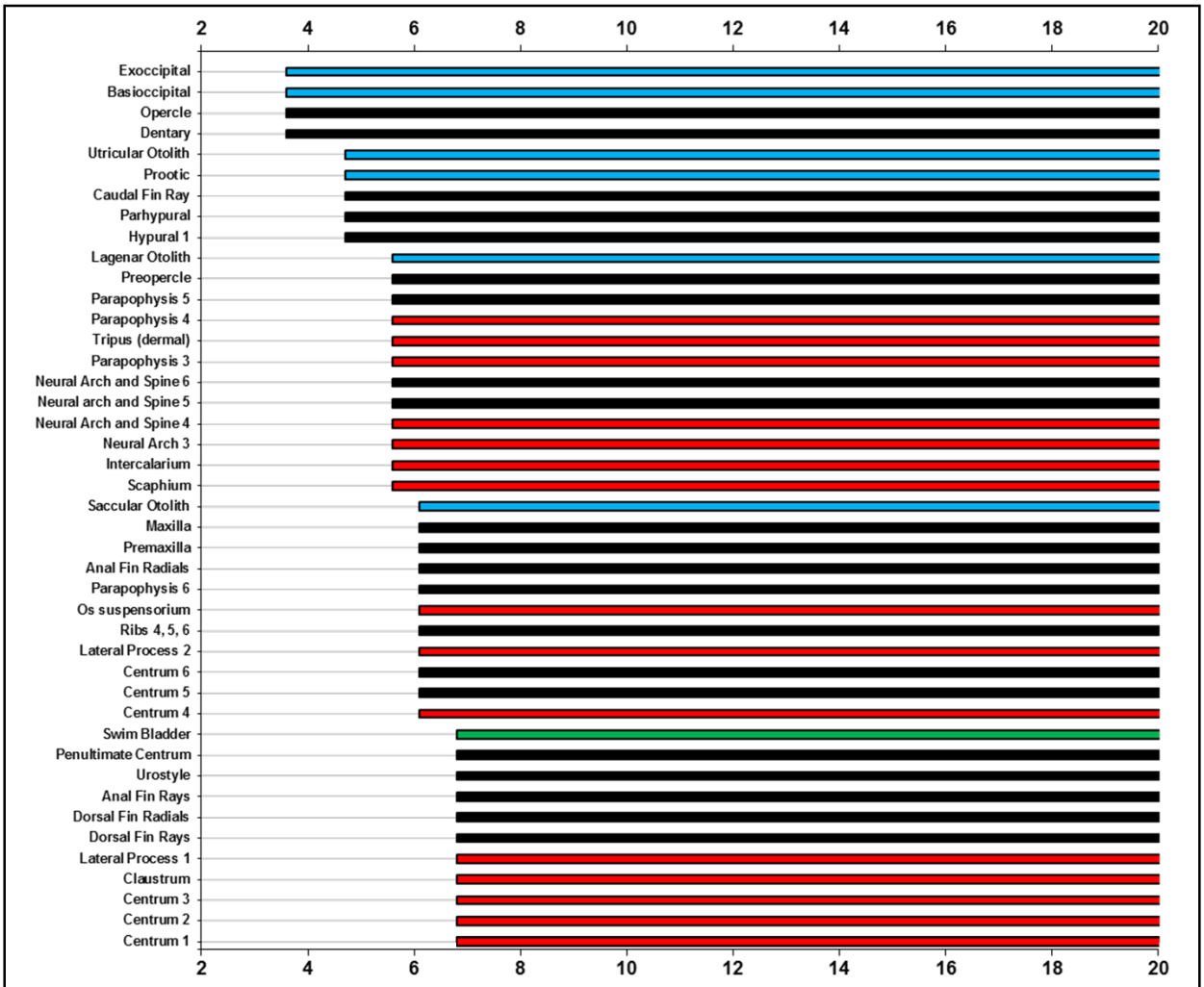

Figure 6. Sequence of development in *Danio rerio*, showing the smallest size at which individual elements are always present, from earliest (top), to latest (bottom). Numbered axis represents size (mm SL). Colored bars represent elements of the Weberian apparatus (blue = ear, red = vertebral, green = swim bladder). Black bars = reference structures.

DISCUSSION

Timing and sequence of development varied qualitatively between *Danio* and *Tramitichromis*. Development in *Danio* began early in ontogeny (by 3.6 mm NL) and proceeded rapidly (all structures present by 6.8 mm SL). This pattern was markedly different to the sequence found in *Tramitichromis*, which began development much later in ontogeny (6.0 mm SL) proceeded slower (all structures not present until 17.0 mm SL) than the rate seen in *Danio* (compare Figures 5 and 6). The differences between species results show *Danio* develops more rapidly compared to the longer process in *Tramitichromis.* Given what is known about the breeding behavior of *Tramitichromis* and related Lake Malawi cichlids (mouth-brooding; Konings 1990, 2007), the relative late development is not surprising since larvae are protected by the mother during early development, with larval and early juvenile *Tramitichromis* remaining in the mouths of their mothers until juvenile stages (Konings 1990, 2007).

With respect to the regions of the Weberian apparatus, clear changes in developmental sequence can be seen in the ear, vertebral column, and swim bladder. For the elements of the ear, all three otoliths formed much earlier in *Danio* (by 6 mm SL; Figure 6, blue) compared to *Tramitichromis*, where they were several of the last elements to form (12-14 mm SL; Figure 5, blue). While the otoliths developed together in both species, the earlier shift in development of all three otoliths in *Danio* suggests the elements may evolve as a group as well, due to functional constraint on auditory function (Schellart and Popper 1992). Additionally, the skeletal components of the otic capsule also developed early in *Danio* (as early as 3.6 mm NL; Figure 6, blue). This was in clear contrast to the elements of the otic capsule in *Tramitichromis*, which did not start developing until 6 mm SL (Figure 5, blue).

Vertebral components of the Weberian apparatus began slightly earlier in *Danio* (5.6 mm SL) than in *Tramitichomis* (6 mm SL). This is different from the vertebral analog structures of *Tramitichromis*. However, development proceeded more rapidly in *Danio* (all vertebral elements present by 6.8 mm SL; Figure 6, red) than in *Tramitichomis* (all vertebral elements present by 9 mm SL; Figure 5, red). Overall, vertebral elements of the Weberian apparatus were scattered among control structures within the overall sequence in both species, and no clear shifts in sequence were found.

The development of the swim bladder occurred much earlier in *Danio*. Inflation of the swim bladder occurred by 6.8 mm SL (Figure 6, green). In stark contrast, the swim bladder did not inflate until 17 mm SL in *Tramitichromis* (Figure 5, green), and was the last structure to develop in *Tramitichromis*. The late timing of swim bladder inflation likely relates to the mouth-brooding behavior in this cichlid species (see above).

After looking at the structures of interest in the zebrafish specimens, comparison suggests that structures that have evolved into the Weberian apparatus in *Danio* share similar developmental timing early in ontogeny. The strong functional advantage otophysans gain via the Weberian apparatus has likely created a new modular unit (stable evolutionary configuration, Wagner and Schwenk 2000). Such modules translate anatomical constraint (required for proper function) into both evolutionary and developmental constraint. Thus, the Weberian apparatus can be considered a new module (= Ear-Bone-Swim Bladder), at least partially independent from other nearby regions or structures (Klingenberg 2005, 2008). The development of these subunits have become independent ("decoupled") from their ancestral functional units (otic region, vertebral column, full swim bladder, respectively), and dependent ("coupled") on each other.

CONCLUSION

Prior to conducting this study comparing *Danio* development to *Tramitichromis*, a synchronous relationship in developmental timing between the vertebrae, anterior swim bladder, and ear in cichlids was not expected for *Tramitichomis*. However, a close relationship in developmental timing of all Weberian apparatus structures in the zebrafish was expected, due to the constraints of maintaining developmental functionality. The data collected showed that control structures of both species developed in a similar sequence. This indicates that our experimental method is valid, and the results for comparison are accurate. Likewise, the data also confirmed the lack of an apparent relationship in sequence of Weberian apparatus homologs in cichlids, and confirmed an earlier shift in developmental sequence of Weberian apparatus structures in *Danio*.

The data support the hypothesis that the Weberian apparatus (modified portions of the vertebrae, swim bladder, and inner ear) form a new developmental module in zebrafish. This study has provided preliminary data towards determining if the Weberian apparatus represents a new developmental module in otophysan fishes. However, because this study was limited to comparing two species, it is not sufficient to fully address the question of whether the Weberian apparatus represents an evolutionary module, or the relative polarity of the shifts (delays versus accelerations) between species.

Future Directions

Future studies should collect additional data on development within several other species, including within Otophysi, as well as in more ancestral species in order to gain a better insight into the nature of the shifts in developmental sequence, and allow for a quantitative statistical

analysis. In addition, analyzing a more in-depth ontogenetic series with fewer size gaps (for example, by every tenth-millimeter versus half or full millimeter) and a more robust structure list (full skeleton versus random structure selection) will allow for greater resolution of developmental shifts. By increasing the scope of species and structures analyzed, a more definitive conclusion can be revealed.

REFERENCES

Berra T.M. Freshwater fish distribution. Academic Press, San Diego, CA. 604 pp.

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.

Cubbage, C.C. and Mabee, P.M., 1996. Development of the cranium and paired fins in the zebrafish Danio rerio (Ostariophysi, Cyprinidae). *Journal of Morphology,* 229*:*121–160.

Dingerkus, G. and Uhler, D., 1977. Enzyme clearing of Alcian blue stained whole small vertebrates for demonstration of cartilage. *Stain Technology* 52:229–232.

Esteve-Altava, B., Diogo, R., Smith, C., Boughner, J.C. and Rasskin-Gutman, D., 2015. Anatomical Networks Reveal the Musculoskeletal Modularity of the Human Head. *Scientific Reports* 5:8298; DOI:10.1038/srep08298 .

Goodrich, E.S., 1930. Studies on the structure and development of vertebrates. London: Macmillan and Company. 485 pp.

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.

Klingenberg, C.P., 2005. Developmental constraints, modules and evolvability. *In: Variation: a central concept in biology*. B. Hallgrimsson and B.K. Hall, eds. pp. 219–247. Burlington, MA: Elsevier.

Klingenberg, C.P., 2008. Morphological integration and developmental modularity. *Annual Review of Ecology, Evolution, and Systematics,* 39:115–132.

Konings, A., 1990. *Konings's book of cichlids and all the other fishes of Lake Malawi*. Neptune City, NJ: TFH publications. 495 pp.

Konings, A., 2007. *Malawi Cichlids in their Natural Habitat*. 4th ed., El Paso, TX: Cichlid Press. 424 pp.

Ladich, F. and Wysocki, L.E., 2003. How does tripus extirpation affect auditory sensitivity in goldfish? *Hearing research*, 182:119–129.

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. 703 pp.

Mabee, P.M., Olmstead, K.L. and Cubbage, C.C., 2000. An experimental study of intraspecific variation, developmental timing, and heterochrony in fishes. *Evolution*, 54:2091–2106.

Nakatani, M., Miya, M., Mabuchi, K., Saitoh, K., and Nishida, M., 2011. Evolutionary history of Otophysi (Teleostei), a major clade of the modern freshwater fishes: Pangaean origin and Mesozoic radiation. *BMC Evolutionary Biology,* 11:177; DOI:10.1186/1471-2148-11-177.

Potthoff, T., 1984. Clearing and staining techniques. In: H.G. Moser, ed. *Ontogeny and systematics of fishes.* Allen Press, Lawrence, KS. pp. 35–37.

Schellart, N.A.M. and Popper, A.N., 1992. Functional aspects of the evolution of the auditory system of actinopterygian fish. *In: The Evolutionary Biology of Hearing.* D.B. Webster, R.R. Fay and A.N. Popper, eds. pp. 295–322. New York: Springer-Verlag.

Smith, K.K., 2002. Sequence heterochrony and the evolution of development. *Journal of Morphology*, 252:82–97.

Wagner, G.P. and Schwenk, K., 2000. Evolutionary stable configurations: functional integration and the evolution of phenotypic stability. *Evolutionary Biology* 31:155–217.

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