ABSTRACT

CHANGES IN MORPHOLOGY DURING THE DEVELOPMENT OF THE HORN AND HUMP OF THE CHINESE CAVEFISH *SINOCYCLOCHEILUS FURCODORSALIS*

by
Michelle Pluviose

The genus *Sinocyclocheilus* endemic to southern China and contains many cavefish species. There are approximately sixty species, of which roughly forty are cave-adapted. Cave *Sinocyclocheilus* are characterized not only by lack of pigmentation and eyes depending on the species, but also by a unique prominent horn on the top of the skull and dorsal hump rostral under the dorsal fin. The horn and the hump are generally considered troglomorphic characteristics, although their functions are still elusive. Here, the development of these distinctive structures in the species, *S. furcodorsalis*, was examined by using micro CT scans. The horn of the fish is supported by outgrowth of the supraoccipital bone, forming a basal ossified shelf and a prominent dorsal spinous process, also known as the neural spine. The hump is a caudal extension of the horn. The micro CT scans of seven fish were examined and positive allometry results exist for skull length relative to the neural spine length. However, other features refuted the hypothesis that heterochrony underlies hump and horn development in *S. furcodorsalis*.

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My family for always pushing me to thrive for success.

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CHAPTER 1

INTRODUCTION

1.1 Development, Heterochrony and Allometry

Development and evolution can be used to study how natural selection has allowed for different morphologies to emerge. The process of growth and morphological change during development occurs at different rates in different organisms. How morphologies are modified during development, by either changes in timing or rate, is called heterochrony (Futuyma 2005). Different rules govern the growth of various parts of the embryo; changes in growth time and rate have been well researched and are some of the mechanisms which give rise to different adult shapes in animals (Schoch 2006). Heterochrony can be observed not only from the earliest stages of embryogenesis but also at any stage of life (Klingenberg 1998).

Heterochrony can result in paedomorphosis or peramorphosis, this occurs when adult features of ancestors found in juveniles or younger stages resembling ancestral adults, respectively (Klingenberg 1998). These evolutionary changes can occur from a change in the period of development through shifts in the time of maturity, changes in the rate of morphological development, or changes in the timing of structural development. As maturity is delayed, the animal can become larger than its ancestor since it remains in the juvenile stage longer, also known as peramorphosis. Progenesis can also occur, leading to a smaller adult form because maturity is reached early. Neoteny, a type of paedomorphosis, is the reduced rate of morphological development resulting in juvenile features in the adult form. Additionally, morphological structures can appear earlier,

known as post-displacement and also another type of paedomorphosis, or later in development, known as pre-displacement.

Allometry is the study of how biological measurements co-vary with each other, either within or among individuals. When traits are measured over developmental time, the relationship is called ontogenetic allometry; when the traits are measured in different individuals at the same time, it is known as static allometry. This thesis examines how morphological structures scale in relation to each other. It is significant for future studies, such as behavioral experiments to correlate the size of the horn with behavioral adaptations.

1.2 Fishes

There are approximately 35,000 extant species of fishes. Fishes come in various shapes and sizes that can be depend on environmental changes. Most fish are ectothermic, covered with scales, and have fins that run along the side of the body. Two paired fins are common for helping with speed and locomotion. They also have gills that are used for breathing, and many have a swim bladder for buoyancy. Anthropogenic disturbances can affect the habitat and biodiversity of freshwater fish making them a highly threatened species (Zhao et al. 2011).

1.3 *Gila cypha* and *Kurtus gulliveri*

A hump similar to *S. furcodorsalis* can be seen in the fish *Gila cypha*, also known as the humpback chub. This endangered fish is found in an aquatic environment known for its sediment-rich soil and rough currents (U.S Fish and Wildlife Service 1990). *Gila cypha* are endemic to the upper Colorado River and although they have a flat shaped head, the

hump is prominent. As with *S. furcodorsalis*, the hump is not sexually dimorphic and there is still abundant amount of undiscovered information on its horn.

Kurtus gulliveri (nursery fish) are found in northern Australia and southern New Guinea and the males of the species have a prominent horn. The fish live in rough coastal waters (Berra 2003). Males have horn-shaped heads in which they carry eggs attached to the supraoccipital crest area. Younger males have very small horns, which grow as the fish matures. The females do not possess a horn. The fish in general is shaped like a hatchet and the forehead has a hump that protrudes anteriorly.

1.4 Caves and Cavefishes

Karst caves, as those in southern China, are formed from rock eroded by weakly acidic water and carbon dioxide that has blended in with the atmosphere. Over a period of time, the water begins to seep into cracks and expands the landform, making larger openings. There are various reasons why the ancestors of cavefishes became isolated in caves, they were either trapped or chose to hide in the openings. To add to this diverse habitat, cavefish also have to adapt to lack of light, scarcity of resources, water pH levels, and two different types of terrain: aquatic and land (Soares and Niemiller 2013).

Cavefishes are interesting creatures surviving in a unique habitat. The species that inhabit caves are limited to resources with practically no light; however, these species succeed even with these challenging obstacles. Not only do cavefish survive in that area but they survive among bacteria, fungi, and bats. Cavefish have adapted to this habitat by evolving traits to maintain a higher fitness. Reduction of pigmentation, eye degeneration,

slower metabolism, and increased life span are some of the key troglomorphic traits that resulted from this subterranean environment (Niemiller and Soares 2015).

1.5 Genus *Sinocyclocheilus*

Sinocyclocheilus live in the karst area of southern China (Liang et al. 2011). Caves give rise to the strange morphologies of their inhabitants; nonetheless many unusual animals survive in this unique environment. In this harsh habitat, cave *Sinocyclocheilus* species have many troglomorphic characteristics, such as loss of pigmentation, extreme sensory evolution, low fecundity, and other unique features (Yang et al. 2016). This genus contains a high species diversity, which has evolved due to their dark subterranean lifestyle (Yang et al. 2016).

There are approximately sixty *Sinocyclocheilus* species with various troglomorphic traits for this single genus (He et al. 2013). They are also disturbed by human activities above ground that create different water chemistry levels, and can directly affect the fish population. *Sinocyclocheilus* live in different parts of the cave with very low amounts of light (Li and Shunping 2009). For example, *S. jii*, and *S. angustiprous* are species found at the surface of a cave, *S. flexuodorsalis*, *S. bicornutus*, and others that live in this subterranean habitat and are also known as troglobites. *S. lateristritus and S. quibenisis* are examples of species that live near the mouth of the cave and are known as troglophiles. *Sinocyclocheilus* troglophiles are considered transitional species because there is a horn and a small eye present. All troglomorphic species have a horn and hump feature, and *Sinocyclocheilus* surface species never have this feature (Figure 1.1).
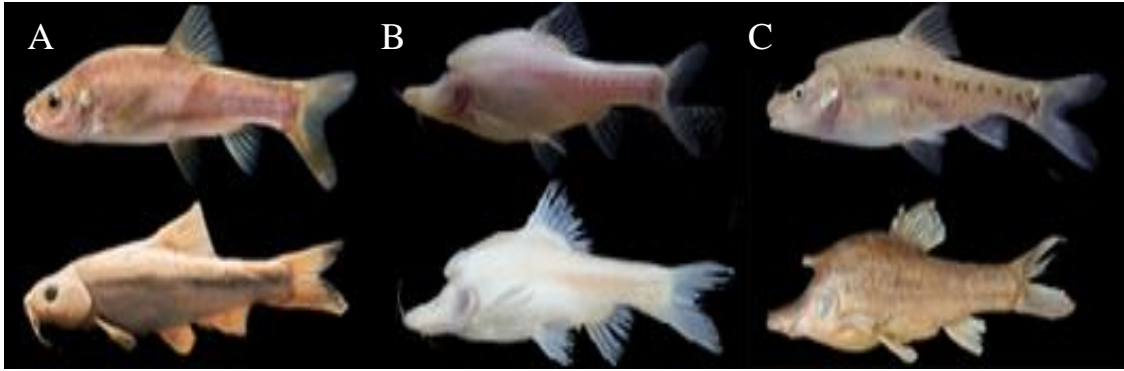
Figure 1.1 Different species of *Sinocyclocheilus* Genus found in the surface (A), cave (B), and transitional (C). Left to right*: S. jii, S. furcodorsalis, S. angularis, S. angustiprous, S. flexuodorsalis, and S. rhinocerous.* Fish are not set to scale and are for illustration purposes only.

This paper focuses on the development of *S. furcodorsalis* (Figure 1.2) through observations of younger specimens to adults in both the horn and spinous processes. We hypothesized that the horn of *S. furcodorsalis* grows nonlinearly and follows a heterochronic pattern.

Figure 1.2 Photograph of an adult *Sinocyclocheilus furcodorsalis* in Dragon cave in southern China. Photo Credit: Yahui Zhao

CHAPTER 2

MATERIALS AND METHODS

2.1 Anatomy

Seven specimens of *S. furcodorsalis* were scanned at the Chinese Academy of Sciences in Beijing, China. All specimens were collected by and stored at the Beijing Museum of Natural History. Specimens were scanned by an Xradia Micro XCT-400 scanner (Zeiss, Germany). The measurements and 3-D reconstructions were created on Mimics 19.0 (Materialise, USA). Two of the three datasets contained three fish scanned simultaneously and one series contained one single fish. All measurements were taken for each fish individually (Table 2.1). For each fish, the following measurements were acquired: the skull (length, width, and height), horn plate (length, width, and height), neural spine (length, width, and height), first vertebra distance to the neural spine, and the angle of the center of horn plate measured (Figure 2.2).

2.2 Calculations

Measurements were compiled and analyzed in Excel (Microsoft, USA). Comparing growth of a structure among fish of different sizes requires allometric scaling. Isometry is measured by comparison to an expected relationship based on the allometric equation:

$$y = ax^b$$

for which data is then log-transformed as

$$\log y = \log a + b \log x.$$

The slope, *b*, demonstrates the scaling relationship and allows for comparison among individuals or species. Both *log x* and *log y* variables in this case were length

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measurements, which predict an isometric relationship with a slope equal to 1 (Figure 2.1).

The first step was to convert the measurements for the scans from pixels to millimeters (mm). Each series of fish had a voxel size of forty-seven microns. Once the measurements were converted to the forty-seven micron conversion factor, they were standardized relative to the total length of the fish. The next step was to log transform the standardized data. For visual comparisons, when graphing a maintained, standard x- axis of skull variables was used. Lastly, a T-test was used to measure statistical significance of slopes from the isometric prediction with +/- 5% difference.
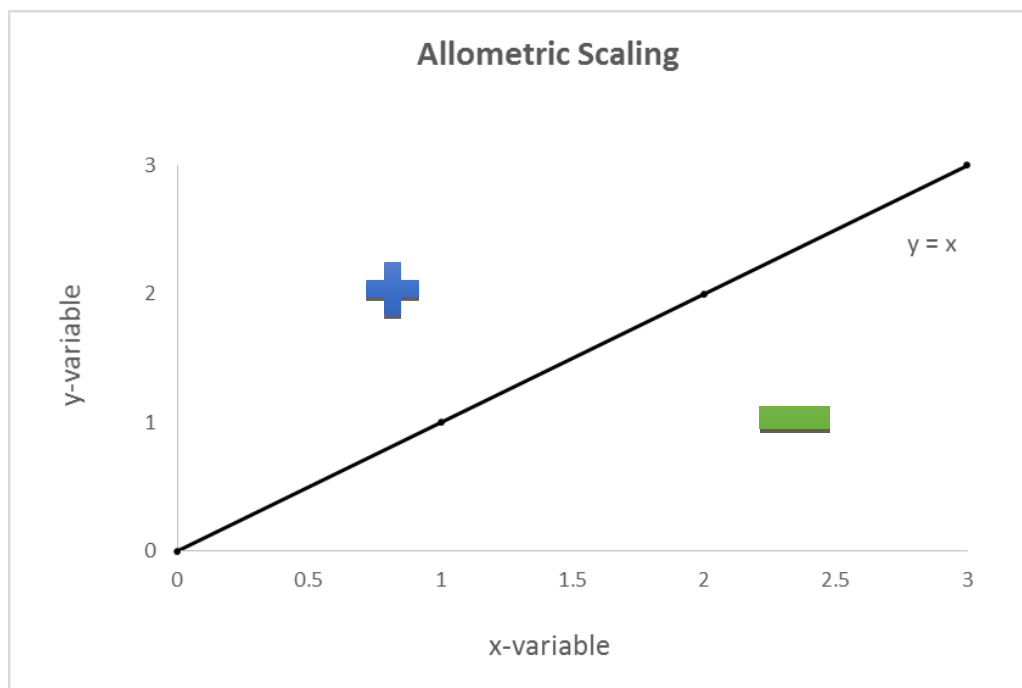

Figure 2.1 Demonstrates allometric scaling with length measurements. Isometric prediction equaling one, a slope greater than one is positive allometry, and a slope less than one is negative allometry.

Table 2.1: Skull, Horn, and Neural Spine Measurements of *S. furcodorsalis*.

Measurements	fish 1	fish 2	fish 3	fish 4	fish 5	fish 6	fish 7
Standard length (cm)	11.58	16.38	16.65	21.51	22.87	25.24	26.98
Skull height (mm)	212.35	282.67	304.39	396.37	352.77	469.34	462.12
Skull length (mm)	348.9	492.97	520.72	687.6	646.9	758.02	810.71
Skull width (mm)	176.91	254.46	238.09	361.15	329.28	398.25	413.05
Horn height (mm)	13.06	39.38	28.89	42.58	53.61	58.61	29.2
Horn length (mm)	67	105.77	115.58	42.78	117.25	164.44	136.73
Horn width (mm)	83.03	116.75	137.87	176.31	149.55	178.85	204.43
Neural spine height (mm)	32.9	70.93	88.81	95.84	90.58	135.27	***
Neural spine length (mm)	17.74	34.09	47.3	58.23	43.72	158.99	***
Neural spine width (mm)	10.98	4.39	7.06	4.06	4.86	4.08	***
Distance to neural spine (mm)	44.29	52.61	74.3	92.84	54.27	94.66	***
Angle of center horn°	117.29	492.97	113.78	646.9	687.6	170.92	114.86

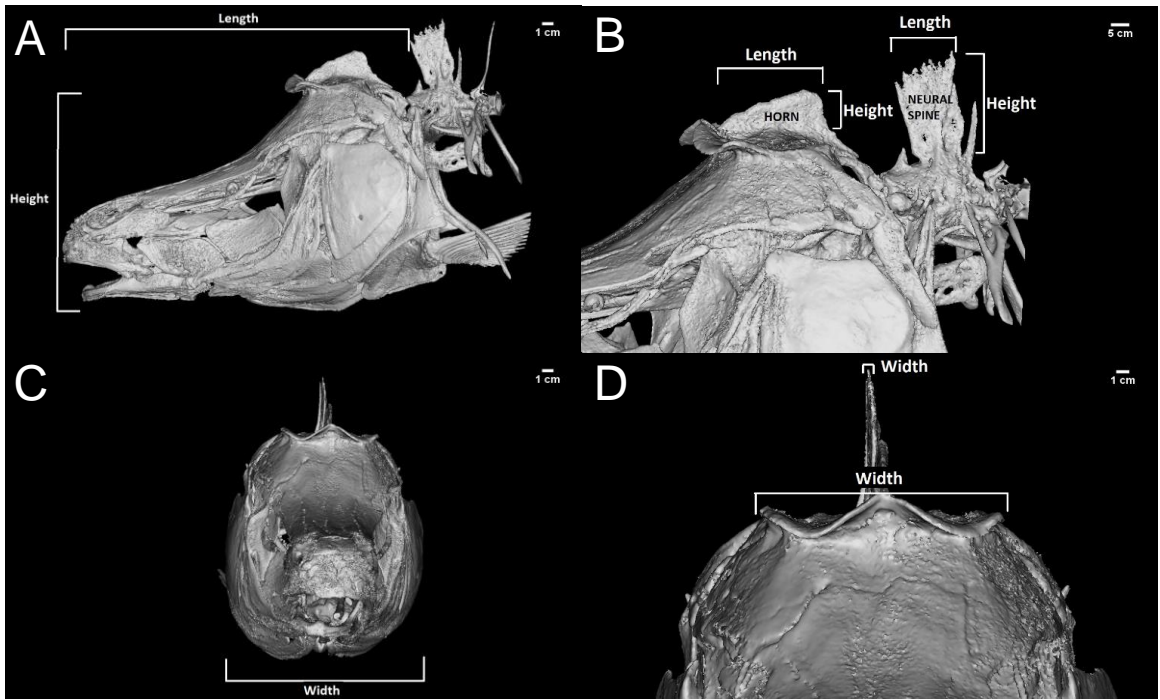Note: ***indicates that the neural spine was misshaped.

Figure 2.2 Measurements used to calculate the length, height (A), and width (C) of the skull. Further measurements of length, height (B), and width (D) of the horn and neural spine.

CHAPTER 3
RESULTS

3.1 Control

The horn of *S. furcodorsalis* is composed of the supraoccipital and parietal bones. Besides the horn, another interesting skeletal structure that grew along with the size of the fish and underlies the hump is the neural spine of the first vertebra (the atlas). Allometric scaling based on growth parameters showed associations between growth of the skull, horn, and the neural spine. Initially, the control is calculated by comparing each feature uniformly. The slope of skull height plotted against the skull length was 0.462, skull height plotted against skull width was 0.412, and skull length plotted against skull width was 0.757. The same method was used for the horn and the neural spine to see structural growth, and to determine if there is an influence on allometric growth. The slope of horn height plotted against the horn length was -0.036, horn height plotted against horn width was -0.066, and horn length plotted against horn width was -0.077. Lastly, the slope of neural spine height versus neural spine length was 1.668, neural spine height versus neural spine width was -1.921, and neural spine length versus neural spine width was -0.833 (Figure 3.1).

3.2 Allometric Scaling

Allometric scaling results were also done for the skull, horn, and hump with the same parameters plotted against different variables. The slope of skull height versus horn height was -1.079, and skull height versus neural spine height was 0.606. In regards to skull length versus horn length, the slope was -5.170, and skull length versus neural spine

length was 2.596. Skull width versus horn width had a slope of 0.419, and skull width versus neural spine width was -3.759. The neural spine was analyzed in the same way - the slope of neural spine height plotted against horn height was 0.539, neural spine length plotted against horn length was 0.076, and neural spine width plotted against horn width was 0.675 (Figure 3.2). This shows that the only positive allometry that existed was skull length vs neural spine length and the remaining were negative allometry.

To measure significant differences of slopes from isometric prediction, we used Student T-test. Results for T-test of skull height versus horn height was 2.89^{-06} and skull height versus neural spine height was 2.42^{-05}. In regards to skull length versus horn length, the result was 4.39^{-05}, and the data for skull length versus neural spine length was 6^{-05}. The slope of skull width versus horn width was 5.86^{-07}, and skull width versus neural spine width was 3.17^{-05}. The neural spine was analyzed in the same way - the slope of neural spine height plotted against horn height was 0.000294, neural spine length plotted against horn length was 0.0528, and neural spine width plotted against horn width was 7.19^{-05}.
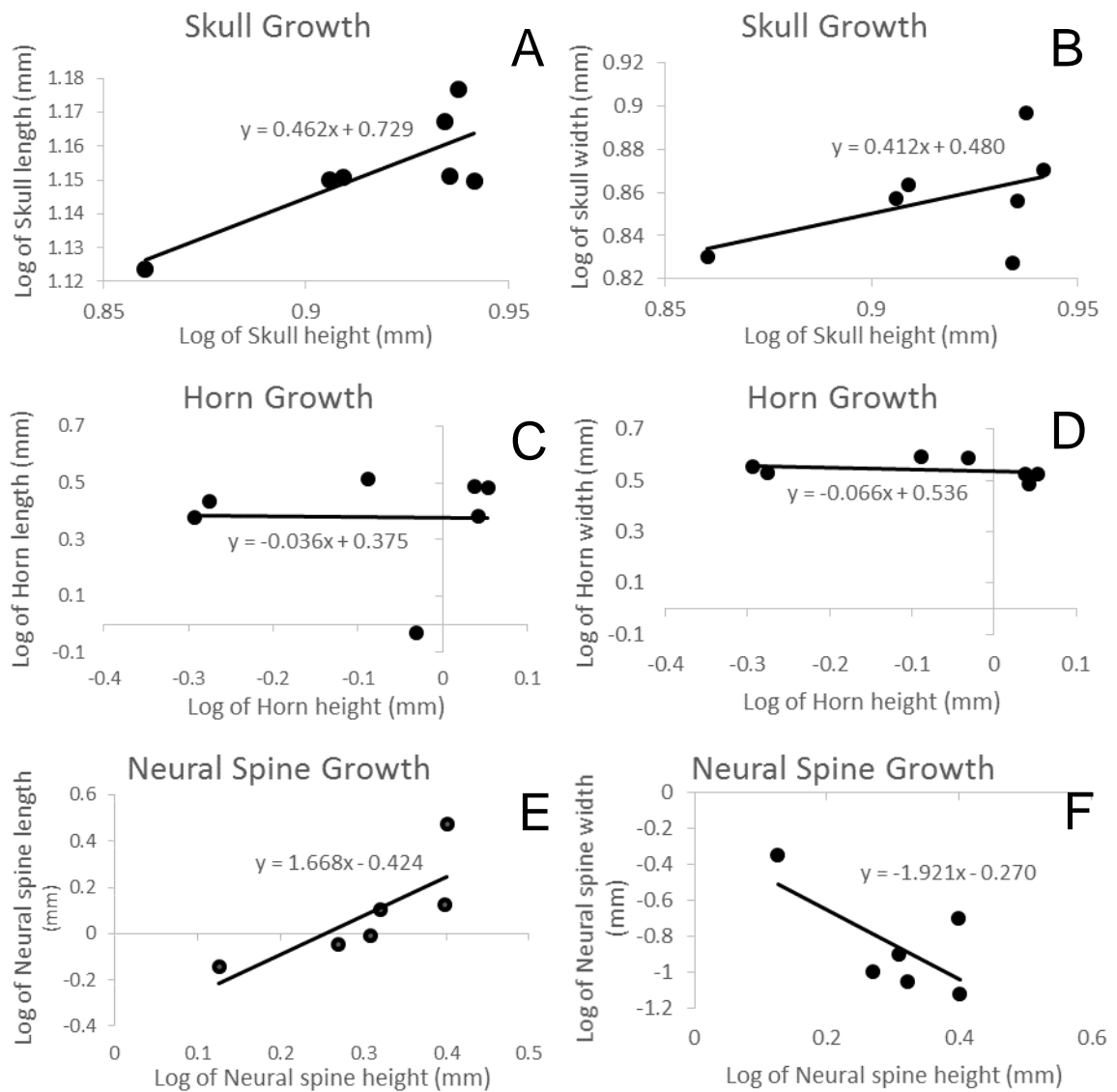
Figure 3.1 Uniform growth between individual parameters for skull, horn, and neural spine. Relationship of skull height vs. skull length (A), skull height vs. skull width (B), horn height vs. horn length (C), Horn height vs horn width (D), neural spine height vs. neural spine length (E), neural spine height vs. neural spine width (F). Represents as a control to understand growth within the structure. **(continued)**
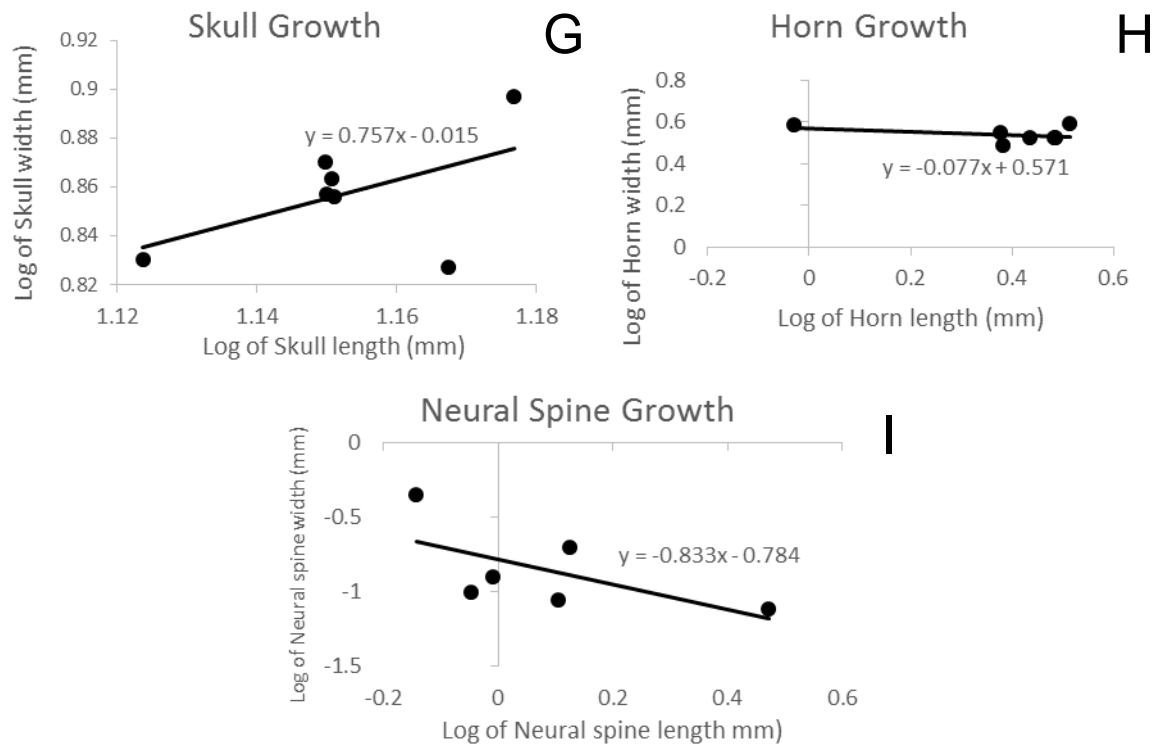
Figure 3.1 (continued) Uniform growth between individual parameters for skull, horn, and neural spine. Relationship of skull length vs. skull length (G), horn length vs. horn width (H), neural spine length vs. neural spine width length (I). Represents as a control to understand growth within the structure.
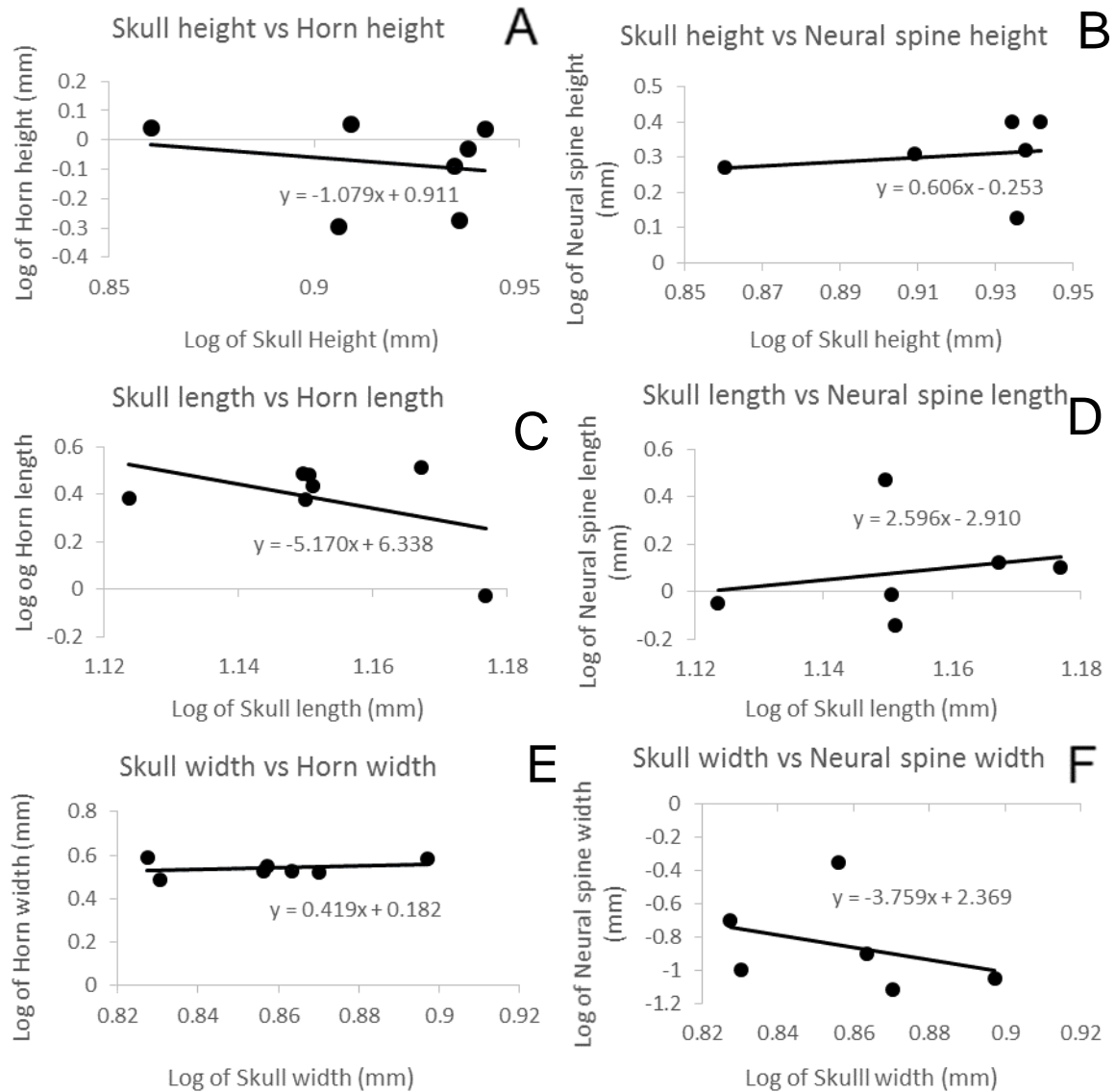
Figure 3.2 Relationship relative to skull height vs horn height (A), skull height vs. neural spine length (B), skull length vs. horn length (C), skull length vs neural spine length (D), skull width vs. horn width (E), skull width vs. neural spine width (F). **(continued)**
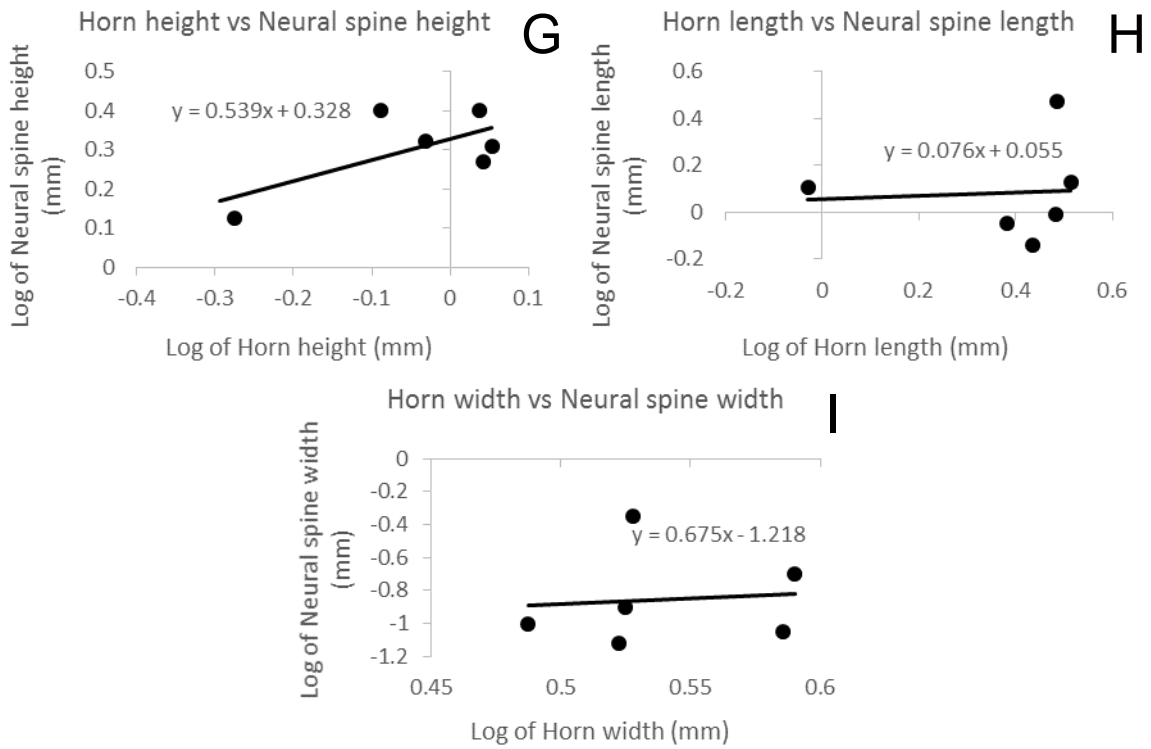
Figure 3.2 (continued) Relationship relative to horn height vs neural spine height (G), horn length vs. neural spine length (H), horn width vs. neural spine width (I).

CHAPTER 4

DISCUSSION

4.1 Summary of Results

Positive allometry results were found for skull length vs. neural spine length. Horn vs. neural spine width, skull height vs. neural spine height, skull vs. horn width, horn vs. neural spine height and length had a negatively allometric relationship. Interestingly, an inverse relationship was discovered between skull vs. horn height and length, and also the skull vs. neural spine width. This relationship identifies that the rate of change of growth for horn height, horn length, and neural spine width decreased precipitously with respect to the rate of change of growth for skull height, skull length, and horn width respectively. This result could be a product of the growth of these structures slowing during ontogeny, indicating a negative correlation. Another speculation could be that these features are shrinking or regressing as the species grows.

The skull, horn, and neural spine were analyzed to determine if they grew uniformly. Compared to skull length, the neural spine length demonstrated positive allometry, which shows that neural spine length increased from its juvenile phase to adulthood. However, in regards to the horn, there were small negatively allometric results when looking at uniformity. This was determined through a slope of -0.036 for horn height vs. horn length, -0.066 for horn height vs. width, and a slope of -0.077 for horn length vs. width.

The neural spine is a site for muscle attachment, specifically the epaxial myomeres. Myomeres are important for feeding because it is important for cranial

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rotation to open the mouth. As the species gets larger there could be larger prey so generation of suction forces would need to be greater to feed. Larger bone with a larger surface area typically support larger muscles. Since in this case the neural spine is getting longer we can speculate that it supports larger epaxial myomere muscles.

A sample set of seven fish is rather small and can skew the overall results of negative calculations for slope. If there was a greater collection of *S. furcodorsalis*, one or two individuals would not change the trend of the graph. Outliers from a larger sample would give an idea whether this feature of trend is for smaller or larger populations or for individuals from a different location. Another issue with a smaller sample size is not being able to see plasticity. In this current sample size there are more medium-sized species so we can see plasticity between certain features. For example, neural spine length seems to have plasticity with medium-sized individuals because it changes results for species with negative growth to a positive. This sample size of seven is not sufficient to determine whether plasticity is found with these features for other sized individuals.

Overall, these results show both refutation and substantiation of heterochrony underlying the horn and hump of *S. furcordorsalis*. The inverse relationship between skull height vs. horn height, skull length vs. horn length, and skull width vs. neural spine width disproves that these features may be growing very slowly or may be shrinking. The hypothesis is proved with the positive allometry results for skull vs. neural spine length.

4.2 Significance of Data

Morphological changes can be related to the fish by understanding the significance in context of its evolution. When comparing this fish to other aquatic species, most

functions are known; however, with *S. furcodorsalis* it remains unknown. Possible functions can be for tissue structure and physiological significance, such as sensory organs or fat storage. Display, energy, movement, and sensory are all reasons as to why the horn may appear as the species develops. However, display for mating is unlikely in *Sinocyclocheilus* because it appears in both male and female individuals. Nonetheless, the absence of sexual dimorphism does not entirely exclude the possibility that the horn and hump may be involved in mating. For example, both males and females of *Cichlasoma citrinellum* have nuchal humps (Bleick 1975). Protection from predators, in addition to protection from head injury can also be a possibility. This data supports the notion that cave-dwelling species accumulate differences because of environmental isolation. *S. furcodorsalis* has a hump and horn characteristic that add to the concept of divergent evolution of cavefish.

4.3 Comparison with Other Fishes

The hump of *Gila cypha* does have the same functional morphology as *S. furcodorsalis*. Functions that may be similar when compared is predation defense. The hump does create a hindrance for swimming (Portz and Tyus 2004). For the species, *Kurtus gulliveri* the horn is used for prenatal care. The egg mass is covered with thick skin, critical for parental care. It is unknown how the fertilized eggs remain on the horn (or sometimes called hook), but the eggs are covered by a gelatinous sticky mixture. Attachments of the eggs have been speculated to stick because of a jagged edge on the anterior portion that keep them together (Berra 2003). Although *K. gulliveri* has a similar morphology to my species of study, the main difference is that in *S. furcodorsalis*, there is no sexual dimorphism in the trait.

4.2 Future Directions

This thesis was a stepping stone to examining the growth of the troglomorphic features of the horn and hump in *S. furcodorsalis*. Until the function of the horn in *Sinocyclocheilus* is described and understood, we will not be able to uncover the pressures that led to this remarkable morphology. A majority of *Sinocyclocheilus* possess horns of unknown function and a large hump on their dorsal aspect behind the head which usually does not consist of any bone. Both traits differ among species. Unique characteristics are considered convergent evolution because they are found in different species as an adaptation (Xiao et al. 2005). A greater collection of additional *S. furcodorsalis* microCT scans will validate these results and determine whether plasticity for the hump and the horn feature exist. Also, to obtain a greater understanding of *S. furcodorsalis* anatomy and behavior experiments can be conducted to provide data on the growth of the hump and horn.

Anatomy: Anatomically, measuring muscle mass of younger to adult species and experimenting on the size of the epaxial muscle to observe feeding habits are some examples of useful data that can be derived from a complementary analysis. Comparative gene expression would also be a fruitful avenue of research. Developmental histology could also help researchers determine whether the cells grow from the bottom and therefore push the new cells upwards or if cells grow from the tip of the horn and add on continuously. Considering that the structure develops at different rates, hormones should also be studied because once they become sexually mature various characteristics develop. For example, growth of nuchal humps in *Cichlasoma citrinellum* have been shown to be inducible with gonadotropin hormones (Bleick, 1975).

Behavioral: Variation of the environment can also be studied, specifically if there are different predators and unstable barriers that cause the horn to function as protection. One behavioral approach would be to test whether the structures would help with other sensory organs since the fish are blind. Another behavioral approach would be to determine if individual growth rates vary by the location of the cave. For example, if ontogenetic habitat partitioning was to take place it may result in different rates of growth in the horn and hump. The horn could also be used for carrying eggs or as a defense mechanism; however, there is not much evidence for this because this feature is found in both males and females. The horn may also be used for protection against the rough subterranean habitat (Yang et al. 2016).

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