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Morphological Correlates of Auditory Sensitivity in the Inner Ear of Two Species of Invasive Carp

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MORPHOLOGICAL CORRELATES OF AUDITORY SENSITIVITY IN THE INNER EAR OF TWO SPECIES OF INVASIVE CARP

A Capstone Experience/Thesis Project Presented in Partial Fulfillment

of the Requirements for the Degree Bachelor of Science in Biochemistry

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By

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ABSTRACT

Silver (*Hypopthalmichthys molitrix*) and bighead (*H. nobilis*) carp, are invasive species that have negative impacts upon ecosystems. *H. molitrix* is known to jump completely out of the water in response to broadband sounds, however, this is not observed in *H. nobilis*. Preliminary experiments reveal that sounds can be used to modify the behavior of carps. Thus, understanding the hearing abilities of these species is important in order to design appropriate acoustical deterrents. Fish heads were preserved in 4% paraformaldehyde and the inner ears dissected and photographed under a light microscope in order to describe the general structure of the ear, which have never been previously described. In addition, some of the ears were processed further, with the sensory epithelia trimmed, and then stained with phalloidin and DAPI, and examined under an epiflourescence microscope. For saccules, hair cell counts were performed in nine 2500 μm^2 locations across the epithelia from rostral to caudal ends. Both species had similar patterns of hair cell densities, with the mean (±SE) number of hair cells found centrally being the least (33.7±3.9 for *H. molitrix*, 32.1±1.4 for *H. nobilis*), and greatest densities found caudally (71.3±7.7 for *H. molitrix*, 75.6±4.8 for *H. nobilis*). At the 55% and 65% locations along the rostral-caudal axis of the saccule, *H. molitrix* had significantly more hair cells than *H. nobilis*. The increased hair cell density in the central saccule of *H. molitrix* may explain why this species is more behaviorally responsive to broadband sounds.

I dedicate this thesis to my parents, Rick and Rita, who are a great inspiration to me.

Also, I also dedicate this work to my brother Blaine, who helped me in the lab.

ACKNOWLEDGEMENTS

I would like to thank Dr. Michael Smith for his mentorship and the opportunity to work in his lab. I also thank Drs. Huskey and Lienesch for sitting on my committee and being mentors to me throughout my collegiate career. In addition, I am appreciative to the United States Geological Survey for supplying the grant that funded this project (G17AC00359), and for what they are doing to protect our environment. I am grateful to Dr. John Andersland for his assistance with microscopy. I would also like to thank the Biology Department, the WKU Biotechnology Center, and the Mahurin Honors College. Finally, I thank my Lord and savior Jesus Christ, for granting me the opportunities I have had during my undergraduate career, and my future aspirations in pursuing a career in medicine.

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INTRODUCTION

As their names imply, Asian carp are not a native species of the United States. They are introduced species, which were brought here in the 1970s to control algal blooms and aquatic vegetation (Nissen et al., 2019). What was as an attempt to aid the water quality in aquaculture facilities and farm ponds, turned into a detriment to the natural environment in the United States, as these fishes escaped and began, and still are, outcompeting local species such as the paddlefish (*Polyodon spathula*), gizzard shad (*Dorosoma cepedianum*), and bigmouth buffalo (*Ictiobus cyprinellus*) (Schrank et al., 2003; Irons et al., 2007; Sampson et al., 2009; Solomon et al., 2016).

The two main species of concern are the silver (*Hypophthalmichthys molitrix*) and the bighead carp (*Hypophthalmichthys nobilis*), both of which are grouped together and called bigheaded carps (Nissen et al., 2019). Due to these fishes being a threat to native ecosystems, removing them from the Mississippi River Drainage Basin, and the numerous other locations they have encroached upon, is a main goal of federal and state fisheries managers. Carps belong to the series Otophysi. Otophysan fishes are known to be particularly sensitive to sound. They have structures called Weberian ossicles, bony structures that mechanically link the swimbladder to the ear. This structure carries the motion of the swimbladder to the ear without attenuation of the signal as a result of distance of travel (Popper et al., 2003; Popper & Fay, 2011; Popper & Hawkins, 2019). It has been hypothesized that this specialization for hearing provided an evolutionary advantage in the shallow freshwaters where otophysan fishes evolved (Ladich & Schulz-Mirbach, 2016).

Acoustical deterrence using intense broadband sounds has shown promise for deterring at least one of the bigheaded carps, *H. nobilis* (Taylor et al., 2005). A unique behavior is observed in *H. molitrix* when exposing them to low-frequency broadband sounds. This behavior is a sporadic jumping with no directionality or trajectory (Vetter & Mensinger, 2016). The discovery of this behavior has led to the development of technology that promotes fish avoidance, like the Bio-acoustic Fish Fence (BAFF) (Lovell et al., 2006). Other methods, as simple as driving a boat with a motor which produces broadband sounds, has also been shown as a way of deterring these fishes. Because of the bigheaded carps' hearing abilities, these low-frequency, broadband sounds appear to disturb the carp, but have minimal effects on most native species (Lovell et al., 2006). In order to better understand how acoustical deterrents may prove useful in removing these species, an in-depth understanding of their hearing and inner ear is needed.

The inner ear is specialized for sound detection and spatial orientation. All gnathostomatans possess an ear with three patches of sensory hair cells: the saccular, utricular, and lagenar maculae which are contained in the saccule, utricle, and lagena endorgans, respectively. In otophysan fishes, structures known as the Weberian ossicles function as an accessory organ for hearing to transmit sound vibrations from the swim bladder to the endorgans of the inner ear (Popper & Fay, 1980). Fish do not possess designated auditory organs like their mammalian counterparts, but rather it has been thought that the utricle plays a primarily vestibular function, the saccule plays a primarily auditory one, and the lagena supports the saccule with roles in both vestibular and

auditory functions, all of which are interconnected by three semicircular canals (Popper & Fay, 1993; Popper et al., 2003; Kwak et al., 2006; Khorevin, 2008).

Within the ear lies three calcareous (i.e., composed mostly of calcium carbonate) structures, called otoliths. Otoliths allow the fish to detect its own movement and signals from its environment when they move relative to how the rest of the body moves. This difference in movement is a consequence of the difference in densities. Otoliths are approximately three times denser than the body of the fish, and thus allows an inertial gradient to be formed to allow the fish to detect motion of the body relative to the head (Popper et al., 2005). The otoliths have their own designations for each endorgan: the utricular otolith is called lapillus(i), the saccular otolith is called sagitta(e), and the lagena otolith is called asteriscus(i).

Each endorgan has an area of sensory epithelia that is referred to as a macula. On this macula is a basal lamina that underlies a layer of non-sensory supporting cells with sensory hair cells interspersed throughout (Oesterle & Stone, 2008; Inoue et al., 2013). In between the supporting cells, there are neurons that extend to make synaptic connections with the hair cells (Szabo et al., 2007; Tanimoto et al., 2009). These hair cells have apical ciliary bundles that consist of one kinocilium and multiple stereocilia that project from the hair cell body into the lumen where they contact the otolith found within each endorgan (Bever & Fekete, 2002; Nicolson, 2005; Cruz et al., 2009; Inoue et al., 2013). Hair cells are either compressed or stretched due to sound waves, and this induces an excitatory or inhibitory stimulus to the auditory nerve, delivering information from the exterior environment to the brain (Nicolson, 2005).

The most diverse inner ear endorgan among fish species is the saccule, in size, shape, and hair cell density. The saccule has been the most fully chacterized as a sound detector in fish species (Popper & Fay, 1999). In addition to this, the saccule is tonotopically organized, meaning that certain areas of the saccule are sensitive to specific frequencies of sound (Smith et al., 2011).

As previously mentioned, there are behavioral differences between both species of carp. Silver carp exhibit a frenzied jumping behavior when exposed to lower frequencies of sound (Vetter et al., 2015; Vetter & Mensinger, 2016), whereas bighead carp do not jump when exposed to these sounds (Vetter et al., 2017). One potential explanation for these behavioral differences is that *H. molitrix* is physiologically more sensitive to sound than *H. nobilis*. In a recent experiment using auditory evoked potentials to measure hearing sensitivity, Nissen et al., (2019) found that *H. molitrix* exhibited greater hearing loss than *H. nobilis* following noise exposure even though their control audiograms are similar. It is unknown if the difference in these species' sensitivity to sound exposure is due to differences in features of the inner ear, such as sensory hair cell morphology or density.

Previous studies show that the density of saccular hair cells in fishes is correlated with hearing sensitivity. For example, female plainfin midshipman, *Porichthys notatus*, increase saccular hair cell numbers during the breeding season when they become more sensitive to the male's advertisement call (Coffin et al., 2012). In addition, noise-induced hair cell loss leads to hearing loss in fishes and post-trauma regeneration of hair cells is correlated with recovery of hearing, as non-mammalian vertebrates have the capability of regenerating lost auditory hair cells (Smith et al., 2011; Smith et al., 2015; Monroe et al.,

2015). The first goal of this study was to describe the general anatomy of the inner ear of bighead carp (*H. nobilis*), as the inner ear of Asian carp species have never been described. The second goal was to determine if the differences in saccular hair cell density between *H. molitrix* and *H. nobilis* may explain differences in behavioral responses (e.g., jumping) to broadband sound.

METHODS

Fish Dissection

Juvenile fish from both species, silver (N=7) and bighead (N=12), were euthanized by an overdose of 0.5% tricaine methanesulfonate (MS-222) via AVMA protocol (2020). Fish heads were removed just behind the operculae and placed in 4% paraformaldehyde for at least a week before being shipped to our lab at Western Kentucky University (WKU), from the USGS Upper Midwest Environmental Sciences Center in La Crosse, WI. *H. molitrix* ranged in mass and standard length from 8.9 to 42.3 g and 10.3-16.7 cm, respectively, while *H. nobilis* ranged in mass and standard length from 4.5-50.0 g and 7.7-17.7 cm, respectively.

Once the fish arrived at WKU, the heads were placed in a phosphate buffered saline (PBS) wash for at least 15 minutes before dissections began. For the examination of general inner ear morphology of *H. molitrix*, the ears were dissected carefully so as to keep the semicircular canals and all the inner ear endorgans intact.

To image the entire ear, a Leica MZ16 stereomicroscope fitted with a Nikon DS-5M camera housed in WKU's Electron Microscopy Facility, was used to take Z-stack images that were then merged for clear images at all focal planes. The ear was placed in a small dish of water in an upright orientation. Approximately 10-20 Z-stack images were recorded for each final "stacked" image. The background of these stacked images were then modified in Adobe Photoshop to make a consistent dark background.

For closer examination of the sensory hair cells of the inner ear epithelia, both right and left saccules, lagenae, and utricles, were all removed from the head and placed

in a microwell with 300 μL of phosphate buffer, being careful to note from which side of the head, left or right, the ear came. Once all the endorgans had been dissected out and trimmed, 3 μL of Alexa Fluor 488-conjugated phalloidin was added to each well and then placed on a shaker to adequately mix the solution with the epithelia. After 15-20 minutes of mixing, the epithelia were mounted on a microscope slide with Prolong Gold antifade reagent with DAPI (4',6-diamidino-2-phenylindole) and a cover slip was placed over the tissue. Fingernail polish was used to seal the cover slip to ensure the reagents and the tissue did not dry out. The tissues were imaged with an epifluorescence Zeiss Axioplan2 microscope (Carl Zeiss, Jena, Germany). Phalloidin labels F-actin, which allowed for the visualization of hair cell bundles.

Epithelia Imaging

Starting at one of the furthest ends—rostral or caudal—the saccule was imaged within the frame of the objective. Multiple images of the entire saccule were recorded using the 20x objective (approximately 50-70 images per epithelium) and AxioVision software (version 2.0) with the Zeiss Axioplan microscope. Once the entire saccule had been imaged, Adobe Photoshop was used to merge the images into the whole saccule, piece by piece. Then nine 2500 μm^2 boxes were overlayed throughout the saccule image from rostral to caudal ends in pre-determined locations (Fig. 5 and 15A). Using the Axio Vision and ImageJ software, individual hair cells were counted within each of those boxes. Hair cells were counted if more than 50% of the hair cell bundle fell within the box. If a hair cell bundle could not be distinguished as multiple cells, it was only counted as one bundle. This process was also repeated for some lagenae and utricles, although the focus of this research was the saccule.

Statistical Analysis

Differences in hair cell bundle density between species were tested using a two-way analysis of variance, with rostral-caudal location and species as dependent variables. Tukey post-hoc tests were used to test for species-specific differences in hair cell numbers at each location on the saccule. SYSTAT version 13 (San Jose, CA, USA) was used for all statistical analysis (α level 0.05)

RESULTS

Inner Ear Morphology

This study was the first to examine the inner ear of bigheaded carps. The ear is located caudally in the skull cavity, just underneath the brain on the lateral, posterior edges. The inner ear endorgans are all attached to one another via the semicircular canals (Fig. 2, 3, and 4). More tightly associated with one another are the lagenar and saccular tissues which share contact with one another through a membranous structure called the sacculolagenar foramen (Bever & Fekete, 2002). When dissecting out the ears and mounting the tissue, because of the close association of these ears, one had to be sacrificed in order to extract the other.

This shape of saccular maculae is common among cyprinids as depicted by the maculae of *C. aurautus* and *D. rerio*, both of which belong to the cyprind family (Fig. 7). This common maculae is also observed for the two cyprinids of this study as well. The saccules for both of these species resembled a propeller shape (Fig. 5 and 6). Beginning at the rostal end, the saccule was very slender and narrow. The first four to five counting boxes (Fig. 5 and 15A, 5-45%) were in this region. At the end of the slender portion, is the centrally located portion, which was very narrow and also demonstrated the least hair cell density for both species (Fig. 14D, 55% location). Immediately following this region, was the other periphery end, the caudal end (65-85% locations), which was shorter in length than the rostral end. In addition to this, the rostral end of the organ possessed a distinctive curve near the end (Fig. 14C). The rostral ends of the saccule were more pointed, whereas the caudal ends were more rounded in shape.

The otoliths of these fish greatly mirrored the shape of their epitheilial tissues. The saccule otoliths were by far the hardest to extract, due to their slender build and fragile nature (Fig. 9A). Very rarely was I able to succefully extract one without breaking it, however, I was able to do this a few times. Otoliths from *H. molitirx*, unfortunately were not saved when examining hair cell densities, and therefore were not able to be examined. However, the otoliths of *H. nobilis* were saved, and therefore can be described (Fig. 9). The saccular otolith is long and narrow, with a sharp, curved hook descending centrally in the otolith. The utricular and lagenar otoliths were easier to extract due to them having a more robust composition. The utricular otolith resembled a bean with a rounded outer edge, with the central part of it exhibiting a concave curvature. The lagenar otolith resembled an upside-down disc.

Sensory Hair Cell Quantification

I examined the differences in the saccule hair cell densities of these two species of Asian carp, to determine if these differences might explain the characteristic jumping behavior observed in the silver carp species. Due to time and resources, only saccule hair cell counts were performed. Hair cell counts were conducted for each box along the rostro-caudal axis of every saccule (Fig. 15A). Hair cell density was highly concentrated in the peripheral regions, with most at the caudal end (71.3±7.7 for *H. molitrix*, 75.6±4.8 for *H. nobilis*), and the least concentrated in the central region (33.7±3.9 for *H. molitrix*, 32.1±1.4 for *H. nobilis*) (Fig.10).

I found that, at the 55% and 65% locations, there was a significant difference in the number of hair cells (Fig. 10). Mean (±S.E.) hair cell bundle densities were 41.9 ± 3.9 and 53.6 ± 3.3 for the 55% and 65% boxes, respectively, for *H. molitrix* (N=7). Mean

(±S.E.) hair cell bundle densities for *H. nobilis* were 32.7 ± 1.7 and 40.0 ± 2.7 for the 55% and 65% boxes, respectively (N=12). *H. molitrix* exhibited significantly greater hair cell densities at the 55% ($p < 0.05$) and 65% ($p < 0.001$) rostro-caudal saccular locations (Fig. 8 and 10). All other locations showed no significance between the two species in terms of saccular hair cell densities ($p > 0.05$).

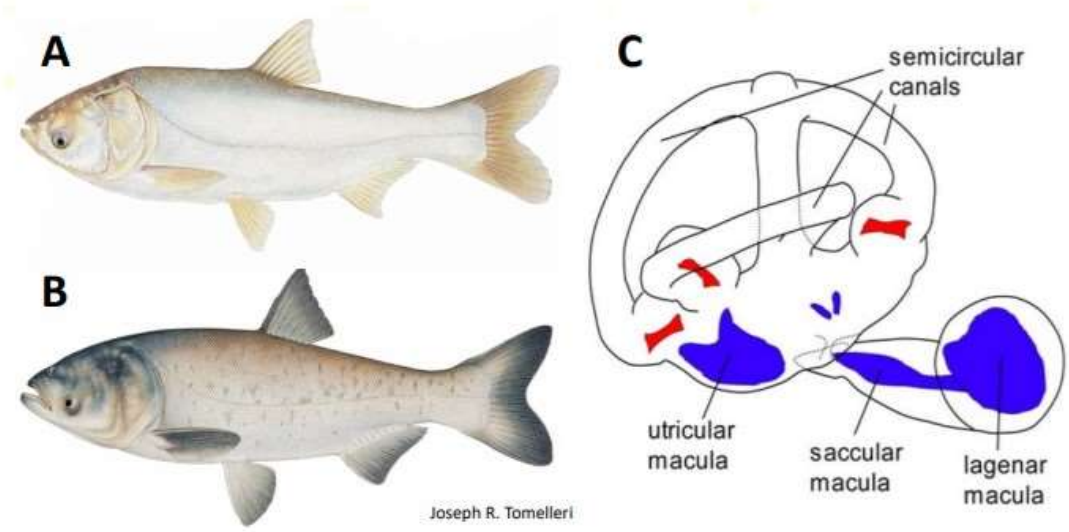

Figure 1: A) *H. molitrix*, the silver carp, B) *H. nobilis*, the bighead carp (artwork by Joseph R. Tomelleri), C) General diagram of a cyprinid inner ear and semicircular canals (modified from Monroe et al., 2015).

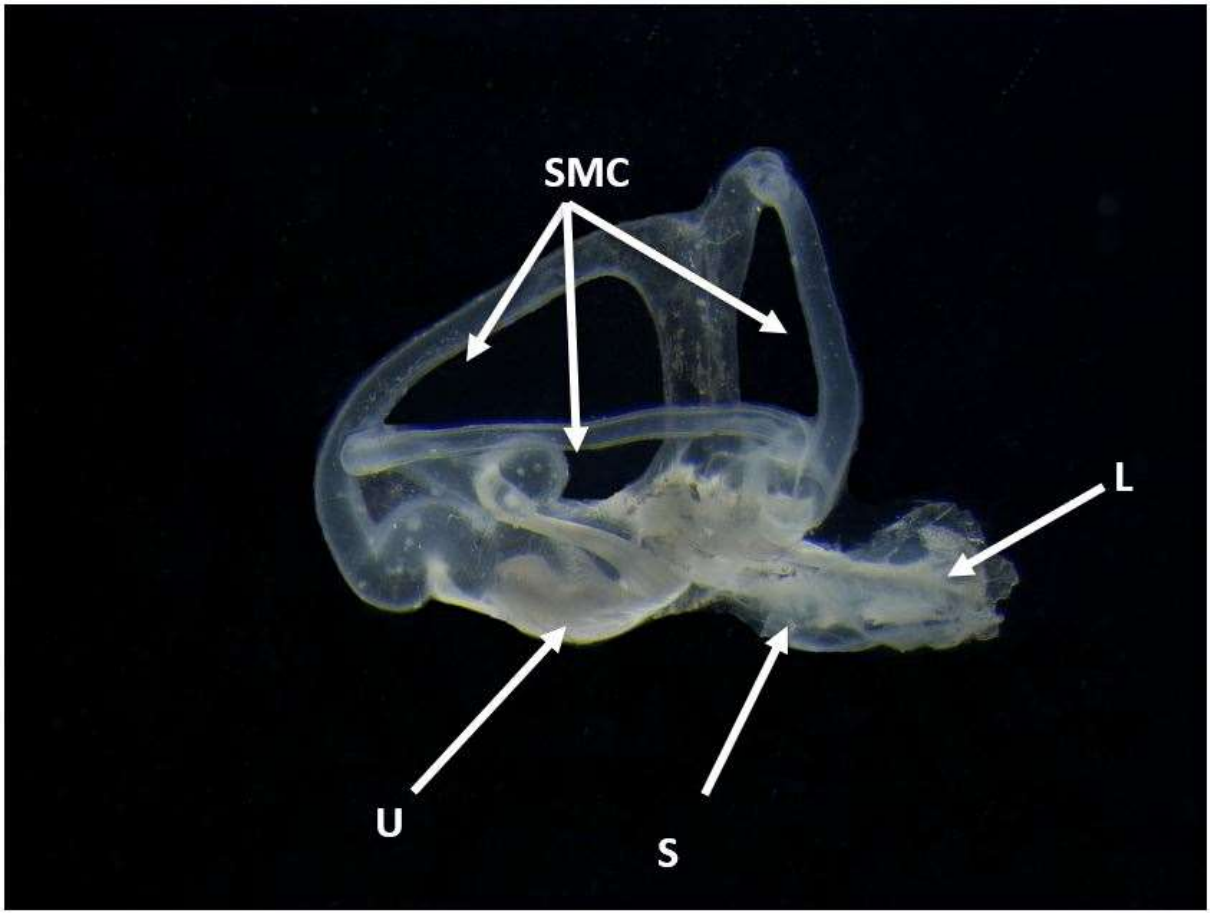

Figure 2: Lateral view of a left *H. nobilis* ear: SMC=semicircular canals, U=utricle, S=saccule, L=lagena.

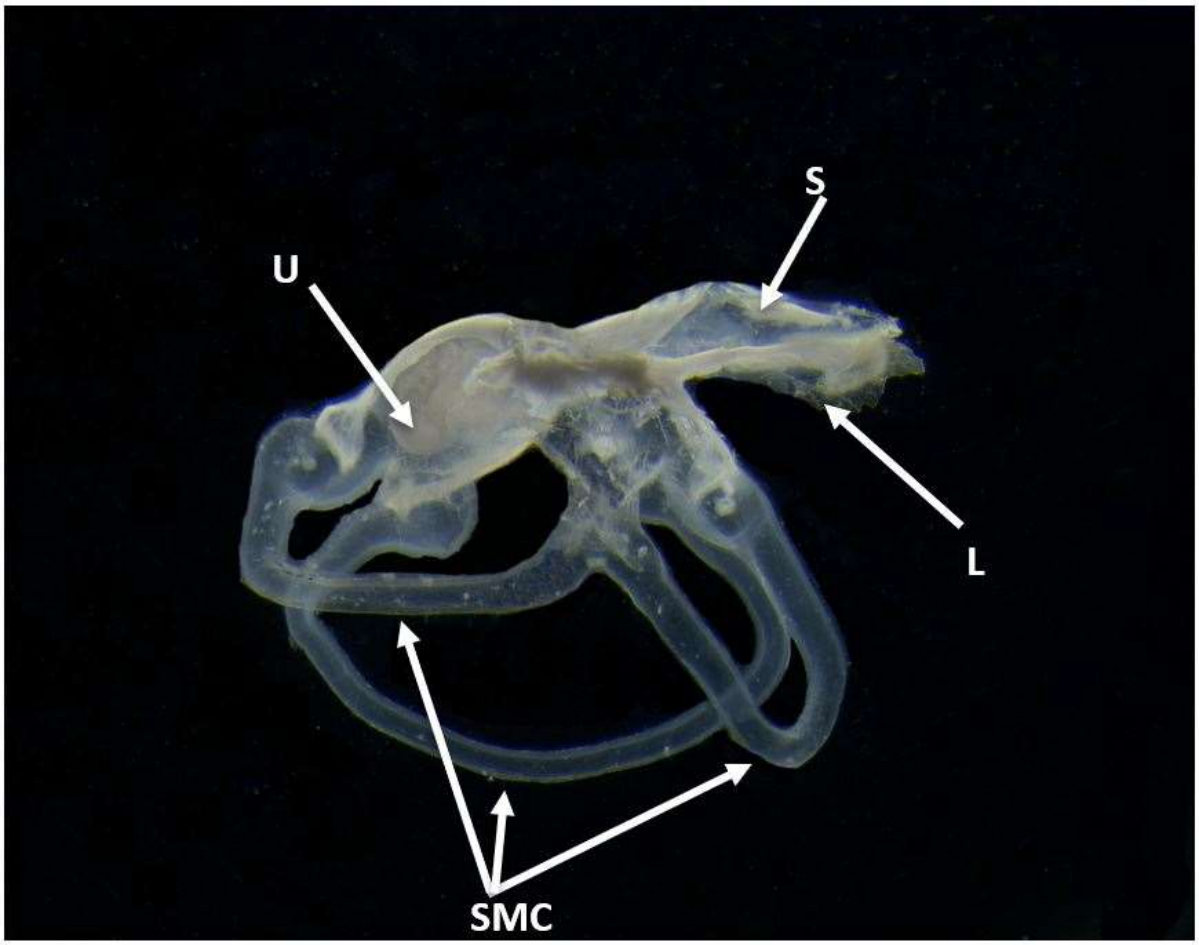

Figure 3: Dorsal view of a left *H. nobilis* ear: SMC=semicircular canals, U=utricle, S=saccule, L=lagena.

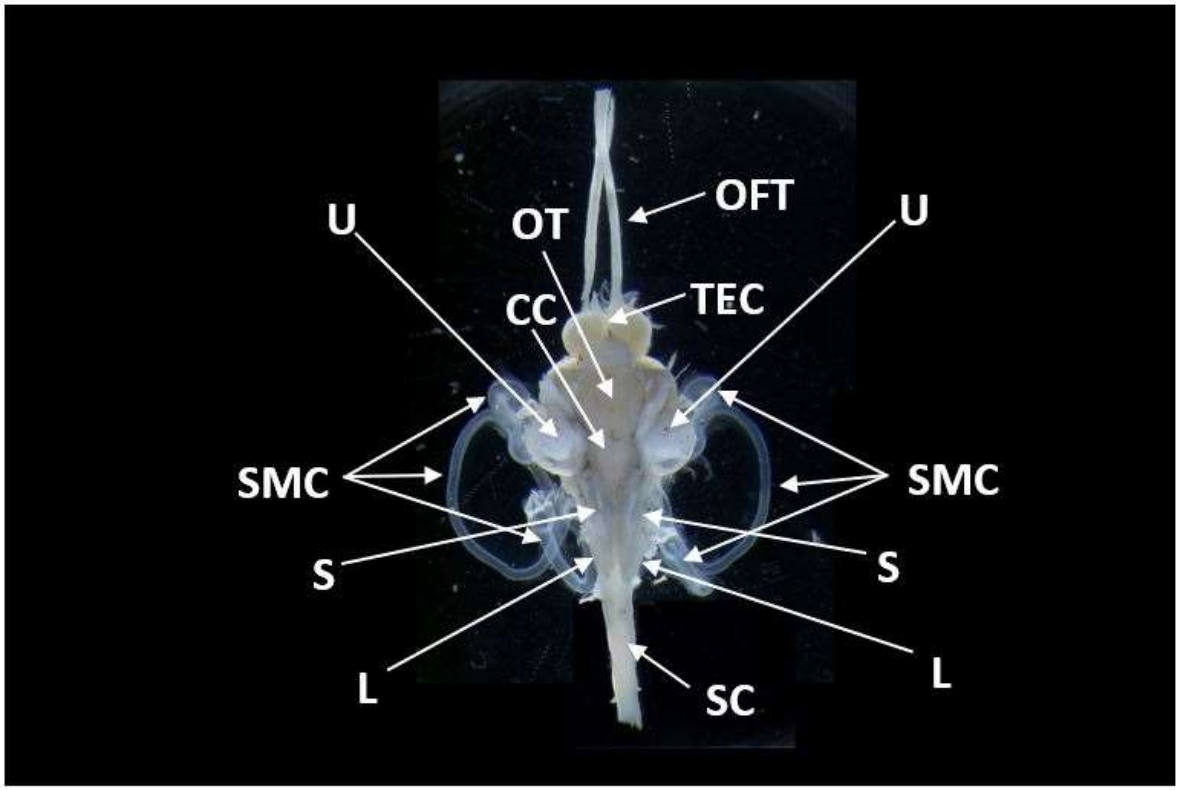

Figure 4: Ventral view of a *H. nobilis* whole brain with both ears. SMC=semicircular canals, U=utricle, S=saccule, L=lagena, TEC=telencephalon, CC=cerebellar cortex, SC=spinal cord, OT=optic tectum, OFT=olfactory tract.

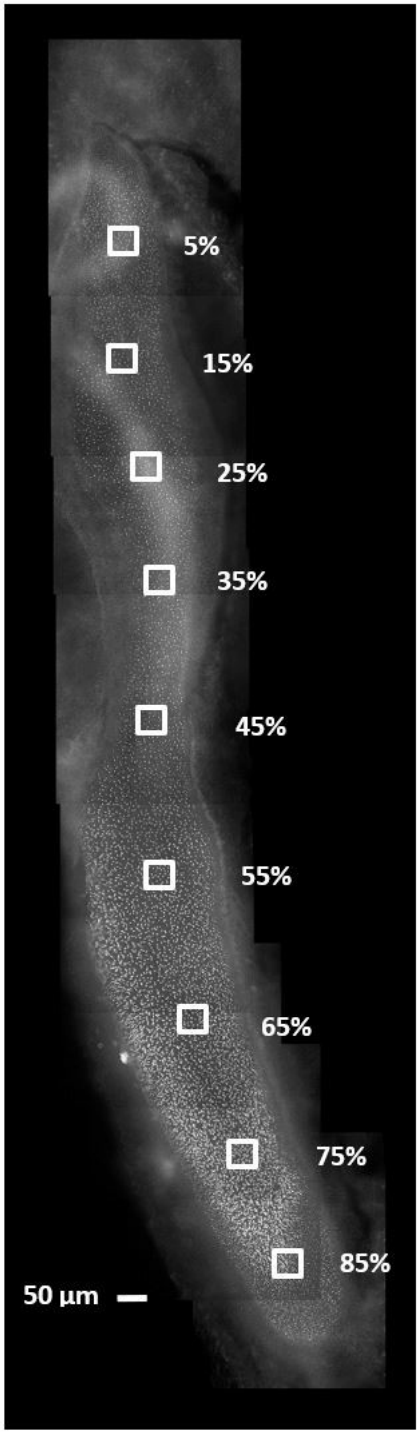

Figure 5: Left saccule from a *H. nobilis* (with nine 2500 µm^2 boxes placed from rostral (top) to caudal (bottom) ends where sensory hair cells were quantified for this study.

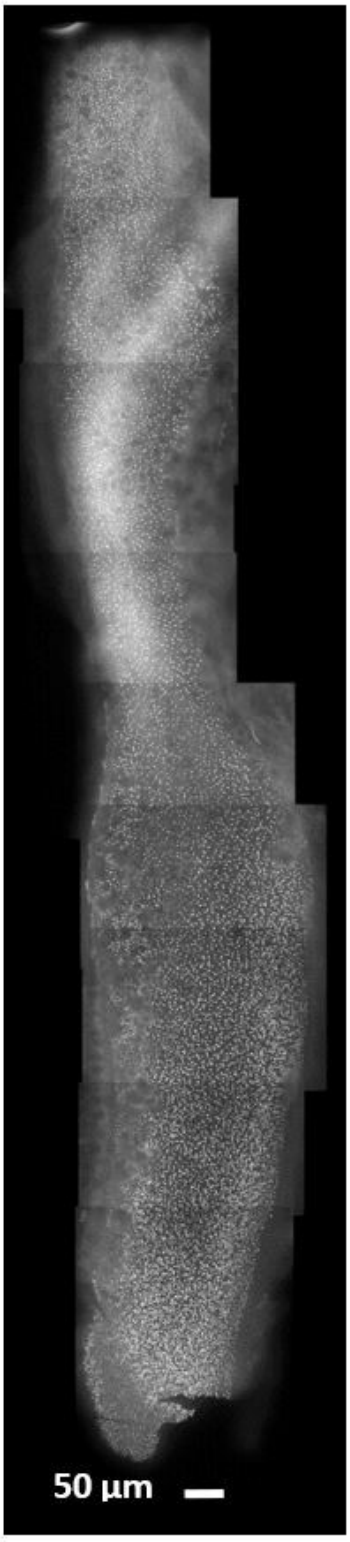

Figure 6: Right *H. molitrix* saccule.

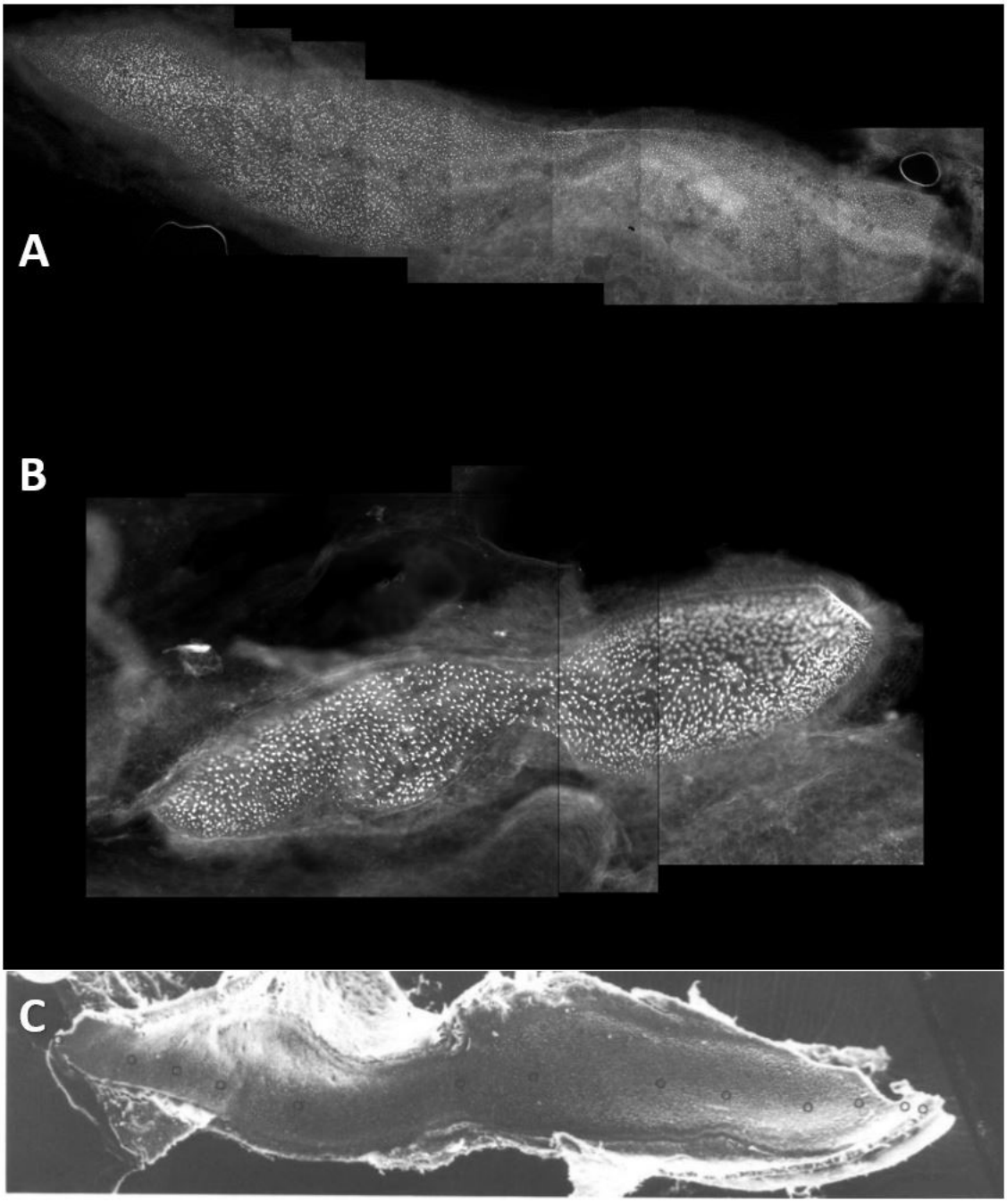

Figure 7: A) *H. nobilis* saccular maculae, B) *D. rerio* saccular maculae, C) *C. auruatus* saccular maculae modified from Platt & Popper, 1984.

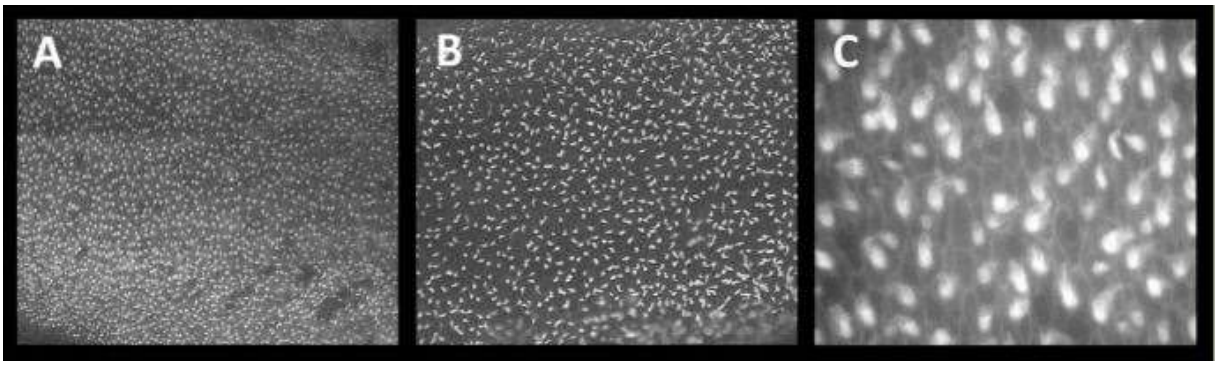

Figure 8: A) *H. molitrix* saccule and B) *H. nobilis* saccule, both at the 65% rostral-caudal location. C) Enlarged image of *H. molitrix* saccular hair cells.

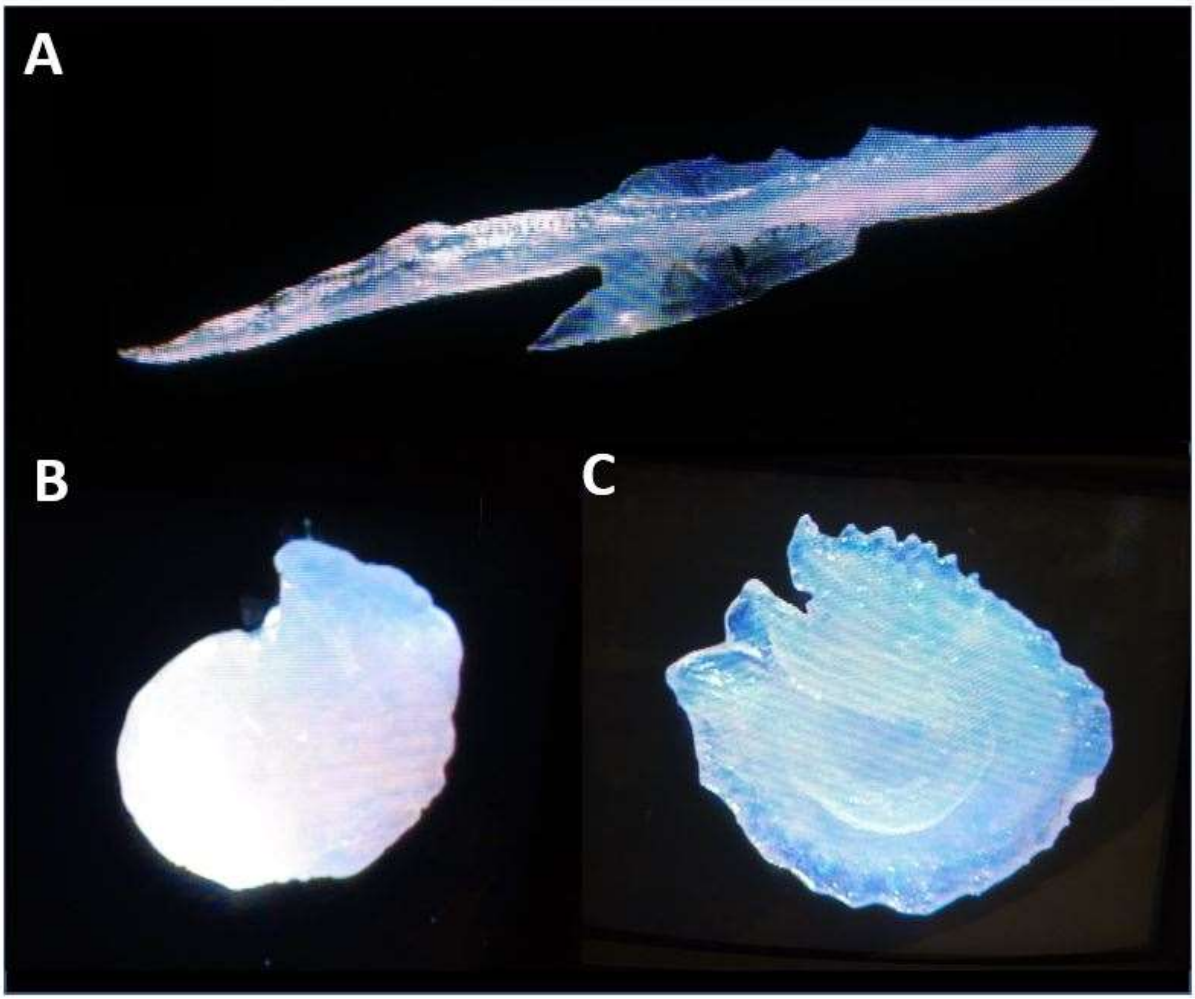

Figure 9: *H. nobilis* otoliths. A) sagitta (saccular), B) asteriscus (utricular), C) lapillus (lagenar).

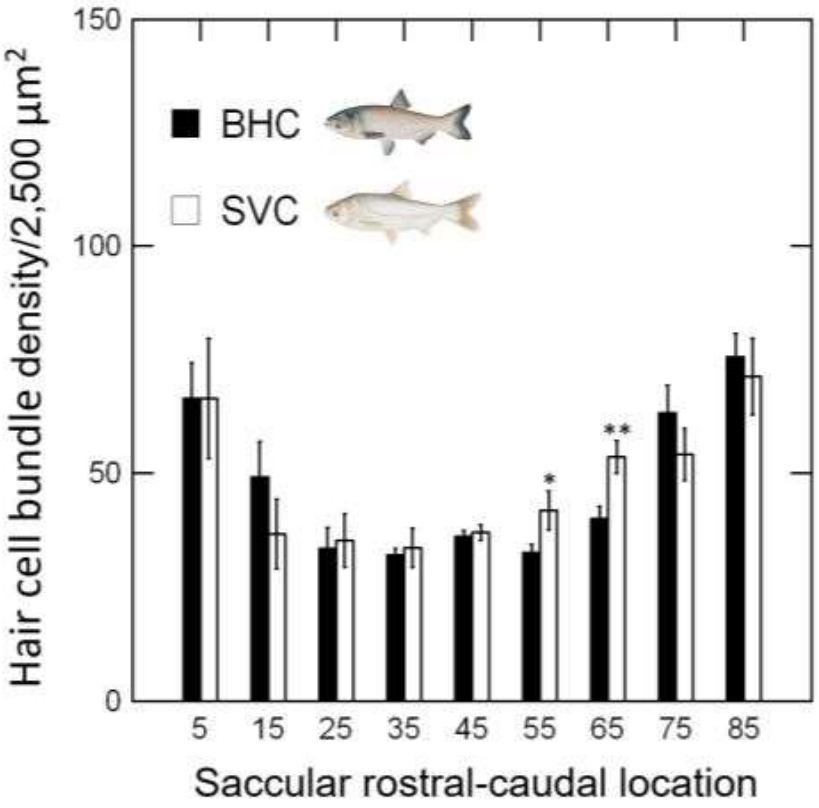

Figure 10: Mean (± S.E.) hair cell bundles/2,500 μm^2 in the saccules of bighead carp, *Hypothalmichthys nobilis* (BHC; N=12), and silver carp, *Hypothalmichthys molitrix* (SVC; N=6-7). Measurements were made at specific rostro-caudal locations along the saccule, with 5% being most rostral and 85% most caudal. "*" $p < 0.05$; "**" $p < 0.001$.

DISCUSSION

Overall Ear Anatomy

Otophysan species show a characteristic similarity in their inner ear morphology which is the interconnection between the saccule and lagena. This interconnection is an opening in the medial wall of the saccular chamber that leads directly into the lagena (Fay & Popper, 1980). I found this characteristic to be consistent with *H. nobilis* and *H. molitrix*, because upon dissection, it was difficult to separate the saccule without damaging the lagena and vice versa. This interconnectedness can be seen in Figures 2, 3, and 4 of the *H. nobilis* ear. For comparison, this similarity can also be seen in goldfish (*Carassius auratus*) which is also a member of the cyprinid family (Fig 11).

The cyprinid family of fishes is a group that includes carps, minnows, and other relatives including the Asian carps, *H. nobilis* and *H. molitirx*, as well as the goldfish, *C. auratus*. The inner ear endorgans are located ventral and lateral to the brain, projecting outward and down in the skull cavity, with an invagination in the skull that surrounds the saccule and lagena (Fig. 4). These fish have several characteristics in common with one another including the orientation and placement of their three endorgans. The saccule and lagena of the carps are attached to one another in a pouch-like structure and only separated by a thin membranous structure, similar to how the goldfish ear is structured (Lanford et al., 2000) (Fig. 11A and 11B, respectively). All three of these fishes have enlarged utricles compared to the saccules. This is a characteristic of otophysan fishes, whereas an enlarged saccule is common amongst non-otophysans (Fig. 11).

An example of a non-otophysan fish is the distantly-related Atlantic molly, *Poecilia mexicana*. This species belongs to the order Cyprinodontiformes. *P. mexicana* possesses a characteristic non-otophysan ear (Fig. 11D), by the enlargement of the saccule. Another distinguishing characteristic among fishes is how the ears are connected. The ears of all fishes are connected by a system of semicircular canals which extend dorsally from the ear endorgans, whether the fish is otophysan or non-otophysan. In the otophysans, like *H. nobilis* and *H. molitrix*, as well as *C. auratus*, this connection spans outward from the utricle. However, in non-otophysans, like *P. mexicana*, the outward extension of the semicircular canals occurs at the saccule (Schulz-Mirbach et al., 2011) (Fig. 11). In addition to this, the ears of *H. nobilis* are positioned so that the utricle is positioned distal and dorsal to the saccule and lagena, whereas in *P. mexicana* the saccule is positioned centrally and the lagena and utricle are positioned proximally and distally, respectively (Fig. 11). The utricle and lagena in non-otophysans are much smaller than the saccules, and in otophysans the saccule is much smaller than the utricle and lagena.

Otolith Morphology

Zebrafish are a close relative to the carps since they too belong to the family Cyprinidae, and therefore were used as one of the comparative species in this study. Compared to zebrafish, the otoliths of the Asian carp were much larger since the carp, even though only juveniles, were considerably larger in body size (Asian carp in the wild can reach weights of approximately 9 kg for silver carp, and 18 kg for bighead carp; U.S. Geological Survey, 2010). Although the otoliths were never weighed or measured, visually there was a distinctive difference (Fig. 9). Having dissected both, the Asian carp

sagitta (Fig. 9A) were more brittle than that of the lapillus or asteriscus, however, the zebrafish sagitta (Fig. 12E) are also extremely fragile and broke almost every time when extracting, due to how small they were. Having worked with both species of Asian carps and zebrafish, as well as observing the common carp saggita (Fig. 12B), it would be logical to predict that all cyprinids possess this very delicate sagitta.

The sagitta of *H. nobilis* has a long, narrow, propeller-like shape. What was interesting about this otolith was the distinctive hook that protruded out of the otolith approximately halfway between the rostral and caudal ends (Fig. 9A). The shape of the sagitta of zebrafish and bighead carp have similarities and differences. Both possess the long, propeller-like shape as well as the distinctive curvature in the middle of the otolith (Fig. 12A and 12E). The zebrafish otolith does not, however, have the distinctive hook that the bighead carp has. Zebrafish saccular otoliths are also much wider at the rostral end compared to the rostral end of the carp (Baxendale & Whitfield, 2016) (Fig. 12E). The carp had more of a pointed tip on both rostral and caudal ends of the otolith, whereas the zebrafish is more blunted on both ends.

It would be advantageous to compare the bigheaded carps sagitta to that of another carp species. One such species is the European carp, *Cyprinus carpio*. The saccular otolith of *C. carpio* has the distinctive curvature in the otolith around the central region (Fig. 12B). However, the hook is much more pronounced and larger compared to that of *H. nobilis*. The common carp also shows a bend dorsally in the otolith going from rostral to caudal ends. The *H. nobilis* shows more of a flattened shape throughout (Fig. 12A). The sagitta from *H. nobilis* and *C. carpio*, do, however, have similar caudal ends. As depicted in Figure 12, the rostral ends for both species are very narrow and smaller in

diameter compared to the caudal and central regions. The caudal end resembles a long, narrow rod. The rostral ends show similarities as well in that they both have a pronounced head-region. This head-region resembles a golf club, and has a slight curvature to it, whereas the caudal ends are straighter throughout.

It has been hypothesized that otolithic size can correlate directly to frequency of sound detected. A theoretical analysis of otoliths in several teleost species suggested that otolith size and the upper limit of hearing capabilities could correlate, with larger otoliths being associated with a narrower range of hearing than a small one might have (Lychakov & Rebane, 1993, 2000; Finneran & Hastings, 2000). Further evidence for this hypothesis was a study done by Popper & Tavolga, (1981) that saw that a marine catfish, *Arius felis*, had very good low-frequency hearing (50-1000 Hz). In contrast to this, *Carassius auratus* and *Ictalurus nebulosus*, had poorer low-frequecy hearing than *A. felis*, but could detect sounds over 3 kHz (Popper & Tavolga, 1981). The difference being that *A. felis* has an exceptionally large utricular otolith compared with other catfish species, leading to the conclusion that larger otoliths function as accelerometers for low-frequency signals (Popper & Tavolga, 1981). This is a very interesting finding, because if density of otolith is a correlation to frequency of sound detected, then perhaps the reason for the thinner sagittae in cypriniforms is for frequency detection of higher frequency sounds.

The otoliths of *H. nobilis* are very different from those of other taxonomic groups of fishes. For example, one study used tomography to examine the saccular otoliths of six species of marine fishes, not closely related to carps. Those species included the Atlantic blue tang (*Acanthurus coeruleus*), white grunt (*Haemulon plumierii*), Atlantic thread

herring (*Opisthonema oglinum*), wahoo (*Acanthocybium solandri*), yellowfin tuna (*Thunnus albacares*), and bigeye tuna (*T. obesus*) (Vasconcelos-Filho et al., 2019). The saggitae of the Atlantic blue tang and white grunt have very similarly shaped otoliths when compared to one another, but very different from those of carps (Fig.12). The marine fishes' saccular otoliths more closely resemble the utricular otoliths of the carp. They are more rounded and bean-shaped. The saggitae of the Atlantic thread herring, wahoo, yellowfin tuna, and bigeye tuna have less of a rounded shape and more so resemble the saccular otolith of the bighead carp than the blue tang and white grunt (Fig. 12). These four species have more of a hook-like structure on them, but they are not as pronounced as that of the carps. One commonality that does exist between all six of these marine fishes is that none of them have a long narrow saggita as found in carps, but rather all of them are more robust and thicker. Perhaps this robustness of their otoliths correlates to the frequency of sound detected (Popper & Tavolga, 1981). Given that none of these fishes are otophysans, it is logical to assume that the range of sounds they can detect are different than that of the otophysans, more specifically the bigheaded carps. Given the thin build of the carp's sagittae it would be logical to assume that they could detect higher frequencies of sound, whereas these six non-otophysan fishes could detect lower frequencies of sound due to their robust sagittae. Thus, there are phylogenetic patterns in otolith shape, with all six of these marine species having very different otolith structures compared to otophysans (especially cyprinid fishes) such as *H. nobilis*.

Saccular Maculae Characteristics

As previously mentioned, the saccule is by far the most diverse endorgan for detecting sound in teleost fishes (Popper & Fay, 1999). The sensory epithelia tissue of

saccules, just as the otoliths that are associated with them, can have drastic morphological differences and similarities, depending upon the phylogenetic relationship of different fish species. Zebrafish have a very similar saccule to that of the bigheaded carps (Fig. 7), but the overall shape and size of the saccule is more similar to that of the goldfish, to which *H. nobilis* and *H. molitrix* are more closely related, than to that of the zebrafish (Chen et al., 2019; Fig. 7). The saccule is very long and narrow in both carp species (Fig. 5 and 6). Both have the characteristic propeller shape, and roughly half-way along the length, the epithelium is twisted. The epithelium is wider on both the rostral and caudal ends compared to the more central parts where it twists.

However, there is a difference at the central region between *H. nobilis* and *H. molitrix* and the central region of *C. auratus* and *D. rerio*. The saccule maculae of *H. nobilis* and *H. molitrix* has reduced curvature in the central part of the maculae compared to the maculae of *C. auratus* and *D. rerio* (Fig. 13). All four species belong to the family Cyprinidae, so it is interesting that the maculae have more of a deeper invagination in *C. auratus* and *D. rerio* and less so in *H. nobilis* and *H. molitrix*. This morphological characteristic could have implications for how the fishes might hear.

Smith et al., (2011) found that noise-exposed *C. auratus* exhibited saccular macula damage at various regions depending on the frequency of sound to which the fish were exposed. However, they did find that across the frequencies of sounds they tested, the central region of the macula always resulted in some damage. This is an interesting finding, because my data revealed that at the 55% and 65% saccule locations, the macula possessed a greater concentration of hair cells in *H. molitrix* than at the same locations in *H. nobilis* (Fig. 10). These differences show that the central region of the saccule might

lead to the reason why *H. molitrix* exhibits the frenzied jumping behavior when exposed to low-broadband sounds and *H. nobilis* does not.

Pattern of Hair Cell Densities

Hair cell densities, when plotted across the rostro-caudal axis along the saccule of both carp species, showed a "U" pattern, with hair cell densities being greatest at the far rostral and caudal ends of the saccule, and the least in the central regions (Fig. 10). Given the morphological similarities of bigheaded carps with goldfish, it would be logical to assume that this species would have a similar pattern of hair cell distribution. Smith et al., (2011) found the same characteristic pattern in goldfish saccules, with hair cell densities being lowest in the center and greatest at the rostral and caudal edges. Another similarity that the carps have with the goldfish is having more cells towards the caudal regions than the rostral. Goldfish have approximately 70 hair cells per 2500 μm^2 at the rostral-most end with 85 hair cells per 2500 μm^2 on the caudal-most end (Smith et al., 2011), and *H. nobilis* in this study had approximately 60 hair cells per 2500 μm^2 at their rostral-most end and approximately 75 hair cells per 2500 μm^2 at the caudal-most end.

Hair Cell Density Variance among Bigheaded Carps

Hair cell densities were distributed throughout the saccule in relatively similar manners in both carp species, except at the 55% and 65% locations (Fig. 10). Here the silver carp possessed greater densities than that of the bighead carp. Mean (± S.E.) hair cell bundle densities for the 55% and 65% locations in *H. molitrix* were 41.9 ± 3.9 and 53.6 ± 3.3, respectively (N=7), and in *H. nobilis* were 32.7 ± 1.7 and 40.0 ± 2.7, respectively (N=12).

These hair cell density differences could have behavioral implications. Both species of bighead carp demonstrate a negative phonotaxis response from human-designed acoustic barriers (Nissen et al., 2019). They also found that silver carp showed the greatest temporary threshold shifts (TTS) following a 24 hour exposure compared to the bighead carp (Nissen et al., 2019). Silver carps appear to be more sensitive to prolonged sound exposure, which might be because of the greater hair cell density in their central saccule. Further, silver carp exposed to 24 hours of sound, did not fully recover their pre-sound exposure hearing ability within 96 hours (Nissen et al., 2019). Another study showed that silver carp exhibited lower hearing thresholds to sounds between 500 and 3000 Hz compared to bighead and common carp (Vetter et al., 2018). These lower hearing thresholds (i.e., higher hearing sensitivity) at intermediate frequencies may have resulted from greater hair cell densities at 55 and 65% rostro-caudal locations of the saccules of silver carp compared to bighead carp.

There is evidence that the saccule of teleost fishes exhibit tonotopic organization. That is, specific regions of the saccule are particularly sensitive to specific frequencies of sound. In an extensive study on goldfish, fish were exposed to specific frequencies of intense sound and then damage to saccular hair cells were assessed (Smith et al., 2011). The hair cell loss was not systemic on the whole saccule, but rather localized to certain areas depending on the frequency of the sound. Goldfish exhibited significant damage in the most caudal regions when exposed to 100 Hz sound, in the most rostral regions when exposed to 4000 Hz, and in the more central part of the ear when exposed to sounds that lie between the two most extreme values (800 and 2000 Hz, respectively; Smith et al., 2011). This data offers evidence about the tonotopic organization of the goldfish saccule.

It is logical to assume that close relatives of goldfish, i.e. bigheaded carps, would exhibit similar saccular characteristics. The density differences between these two carp species exist at the 55% and 65% locations, both of which lie more centrally within the saccule. Using the tonotopic map of goldfish (Smith et al., 2011), it would be predicted that these saccular locations in carp would be sensitive to sounds ranging between 600 and 1000 Hz. These frequencies are also where carps and other cyprinids, like goldfish and zebrafish, are most sensitive to sound pressure (Smith et al., 2011; Monroe et al., 2016; Vetter et al., 2018).

H. molitrix appear to be more behaviorally sensitive to complex tones, like that produced by an outboard motor, than they do to pure tones. *H. molitrix* showed slightly more deterrence responses (0.18 ± 0.06) when exposed to 2000 Hz than they did when exposed to 500 Hz (0.13 ± 0.06), both of which were pure tones (Vetter et al., 2015). On the contrary, when exposed to complex broadband tones, i.e. recordings of an outboard motor, the carps responded during 100% of the complex trials with an average of 11.8 ± 1.3 (range 3-37) consecutive responses per trial. The behavioral difference between the two carps is seen in response to the sounds produced by an outboard motor. Although both demonstrate negative phonotaxis when exposed to pure tones, the sound of an outboard motor (0.6-10 kHz) causes *H. molitrix* to demonstrate the characteristic behavior of jumping as a response (Vetter et al., 2015; Vetter & Mensinger, 2016; Vetter et al., 2018). Most of the energy dissipated by an outboard motor is done so as intermediate frequencies (Vetter & Mensinger, 2016). This would stimulate the centrally located regions of the saccule. Since silver carp have a greater density of hair cells at the

central locations (55 and 65%) than bighead carps, this may be why they are more behaviorally responsive to these sounds.

Implications for the Ongoing Asian Carp Invasion

At the end of the day, our main priority has to be our local environment and ecosystems. These carps have caused quite a few problems already, and have the potential to do a lot more if we do not do something. Barriers such as the Bio-Acoustic Fish Fence (BAFF) (Lovell et al., 2006), have already been implemented as means to try and slow these species' invasion, but more should be done. Something that could possibly help is to simply drive a boat across the lakes where they have invaded, with speakers attached to the front of them that are producing these low frequencies. Even if a speaker is not available, simply driving the boat is enough to cause negative phonotaxis behavior. These are just simple techniques that may work to deter these fishes from a specific area, but may not be effective at removing them from the body of water. This is why more research is needed on the bigheaded carps hearing abilities. In this study, the saccule was the only endorgan investigated due to time and resources, but in order to gain a more holistic understanding as to how they perceive the acoustical world around them, we must gain a more in-depth understanding of the maculae of the lagena and utricle. By doing so, we will gain more insight as to how they hear, and this might lead to the development of more effective acoustical deterrents. One thing that is for sure, is that something should be done to migitate the environmental damage caused by these fishes, as the responsibility to protect and improve our ecosystems is on us all.

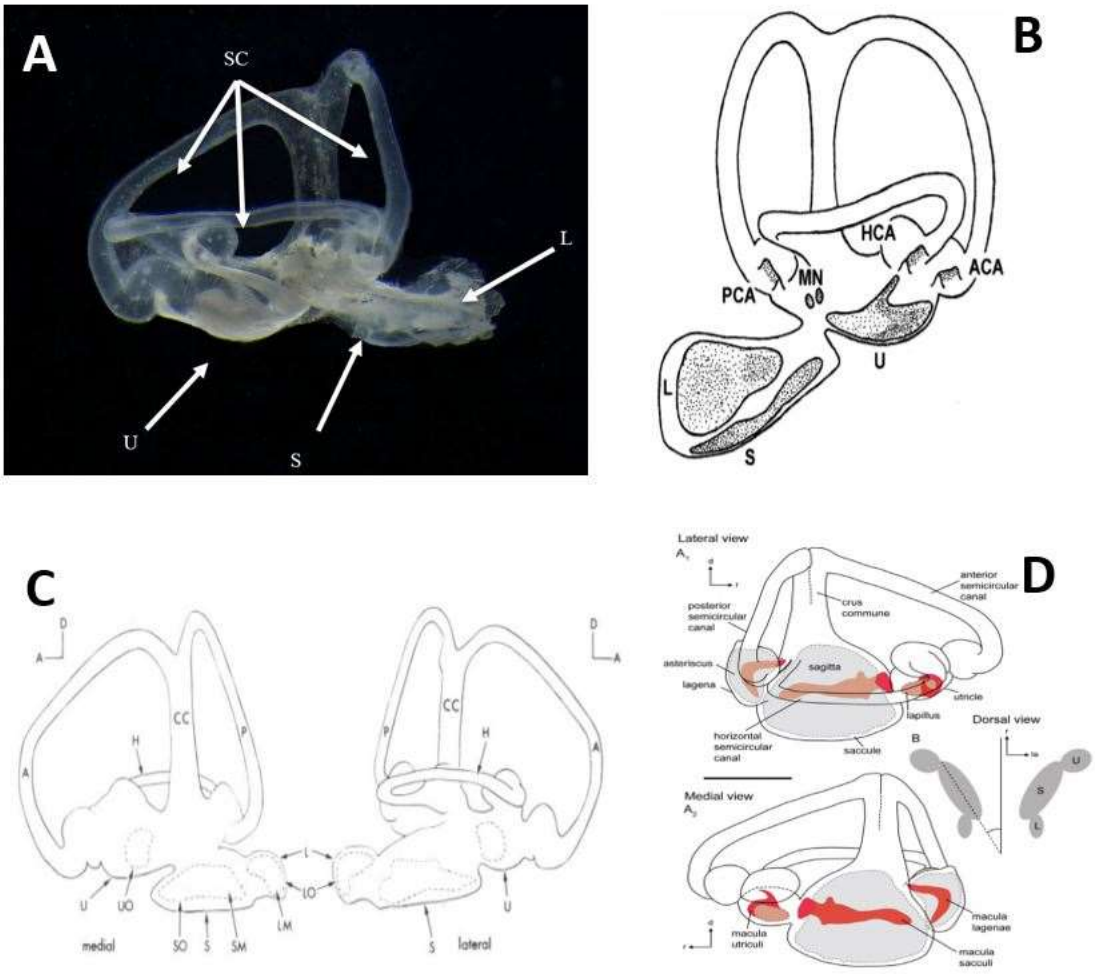

Figure 11. A) *H. nobilis* inner ear lateral view, B) *C. auratus* inner ear lateral view modified from Lanford et al., 2000, C) Non-otophysan fish ear, genus *Sarda*, modified from Popper, 1978, D) *P. mexicana* inner ear lateral and medial views, modified from Tanja Schulz-Mirbach et al., 2011.

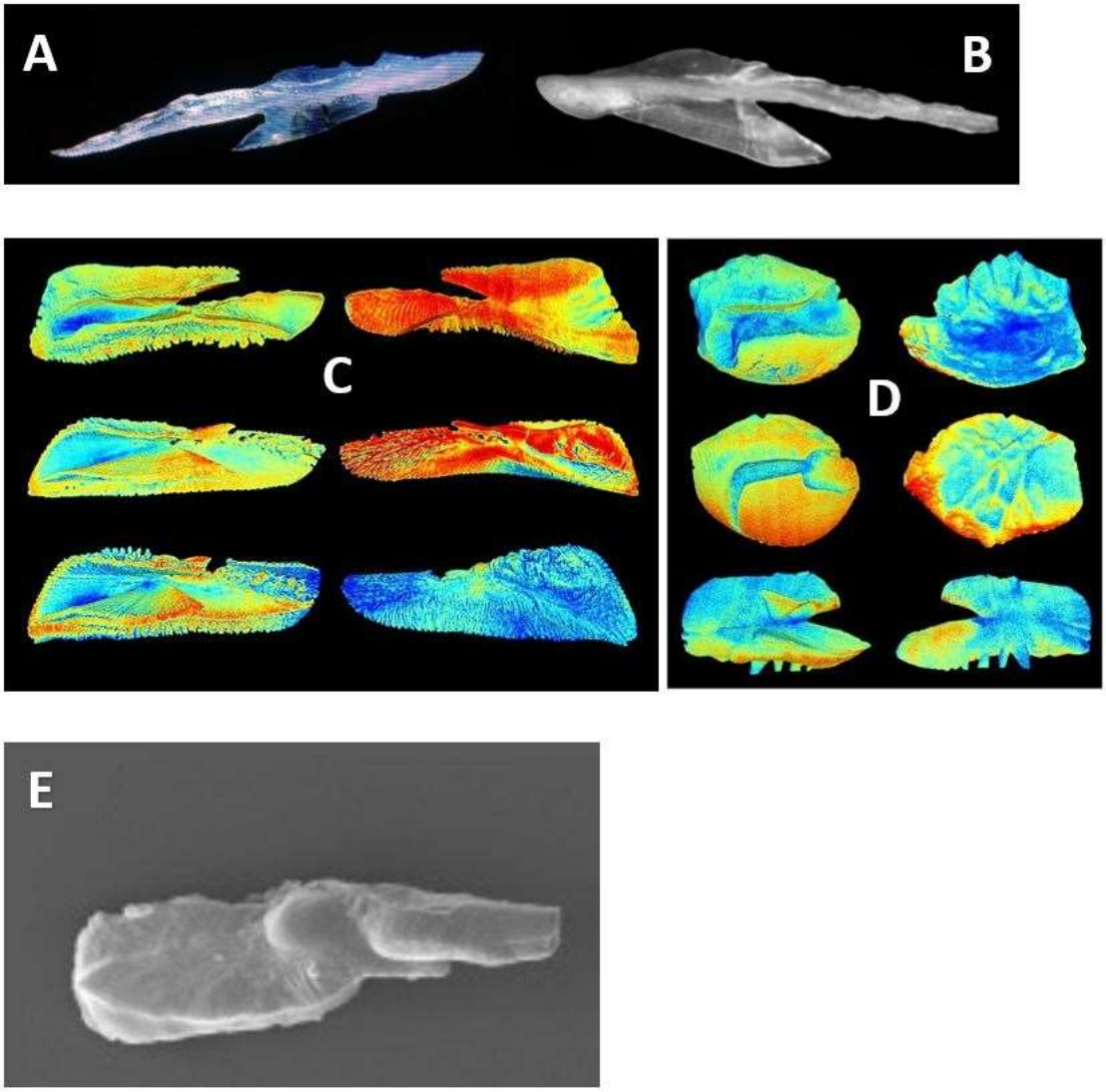

Figure 12. A) *H. nobilis* sagitta, B) *C. carpio* sagitta (modified from Schulz-Mirbach, & Reichenbacher, 2006), C) External (left) and inner (right) views of sagittae from *A. solandri, T. albacares, and T. obesus* (modified from Vasconcelos-Filho et al., 2019), D) External (left) and inner (right) views of sagittae from *A. coeruleus, H. plumierii, and O. oglinum* (modified from Vasconcelos-Filho et al., 2019), and E) *D. rerio* sagitta (modified from Baxendale & Whitfield, 2016).

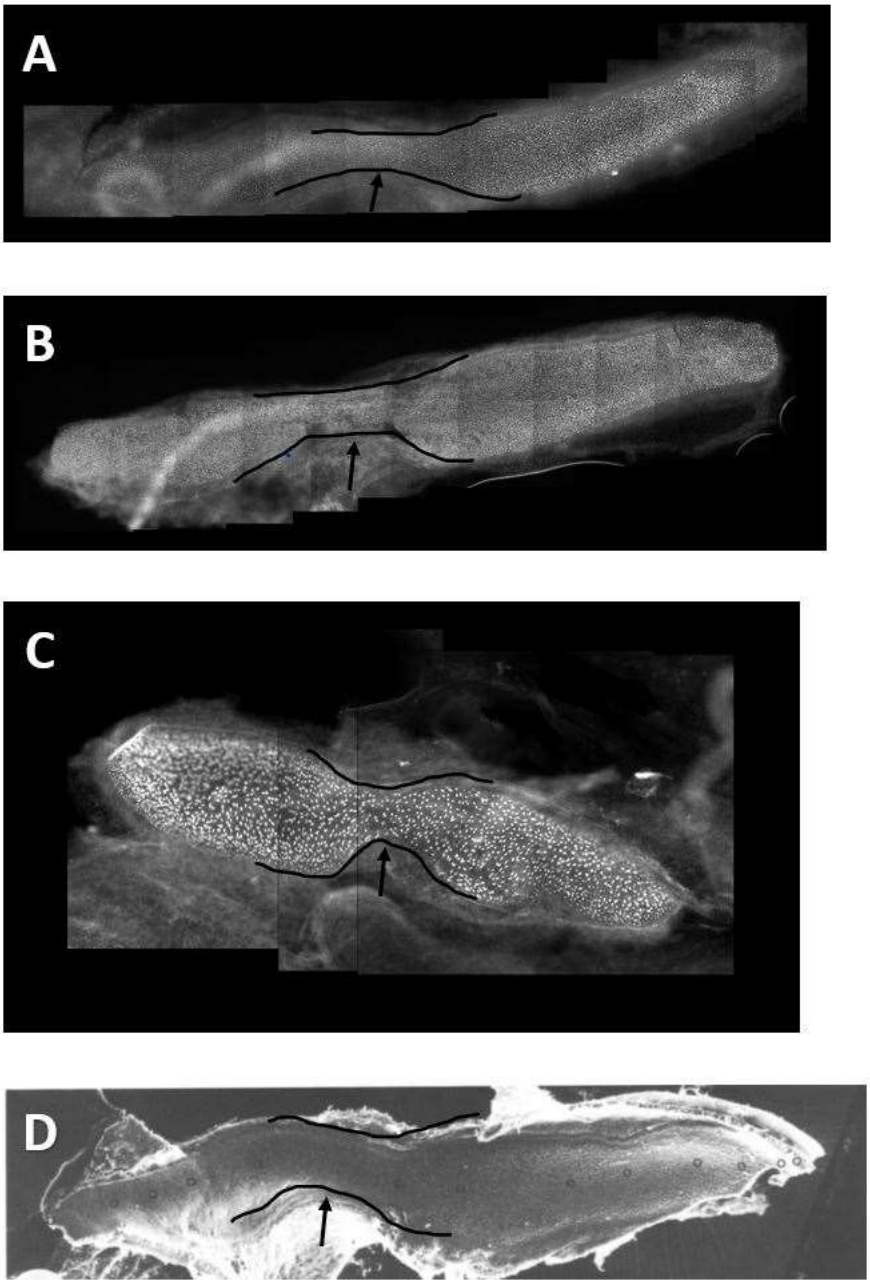

Figure 13. A) Saccular maculae of *H. nobilis*, B) *H. molitrix*, C) *D. rerio*, and D) *C. auratus* (modified from Platt & Popper, 1984). Drawn black lines and arrows highlight the narrow neck separating rostral and caudal regions of the saccule.

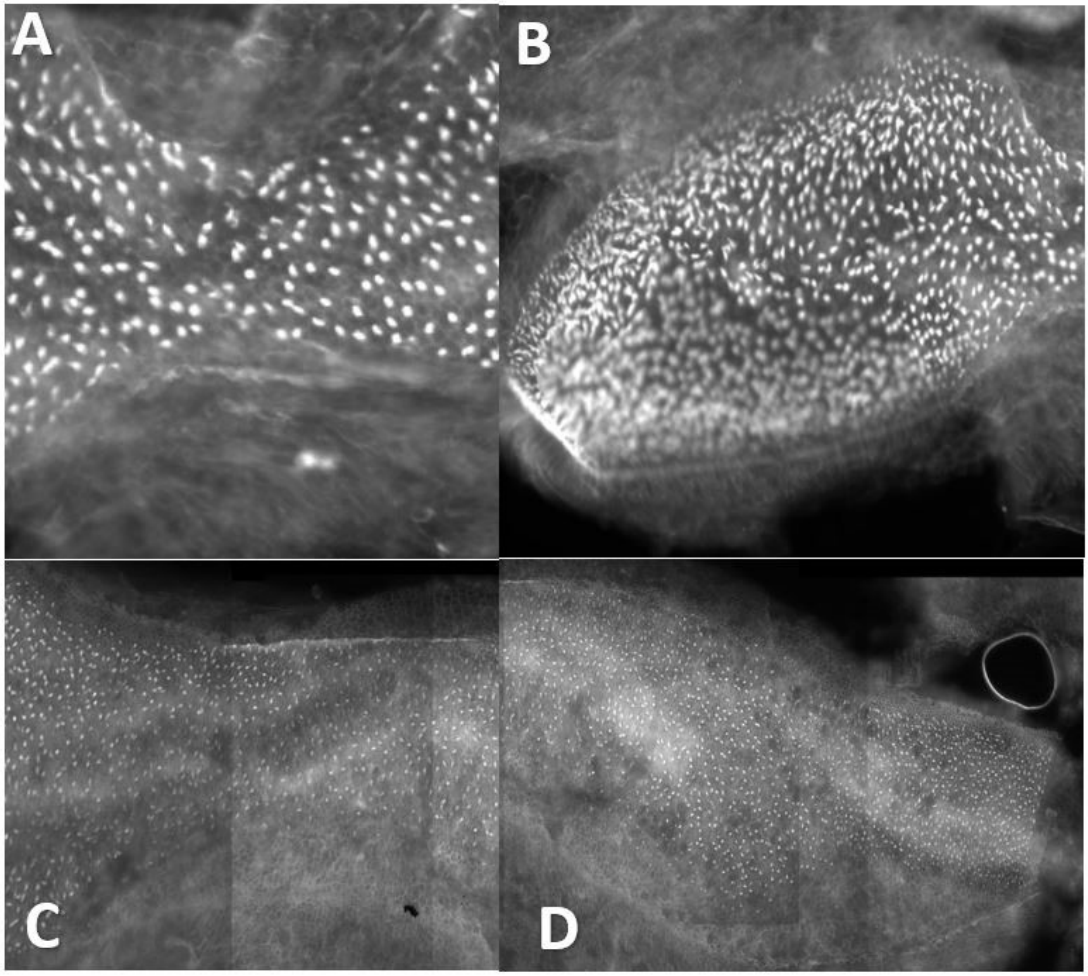

Figure 14: Zebrafish central hair cell density A), zebrafish rostral hair cell density B), bighead carp central hair cell density C), and bighead carp rostral hair cell density D).

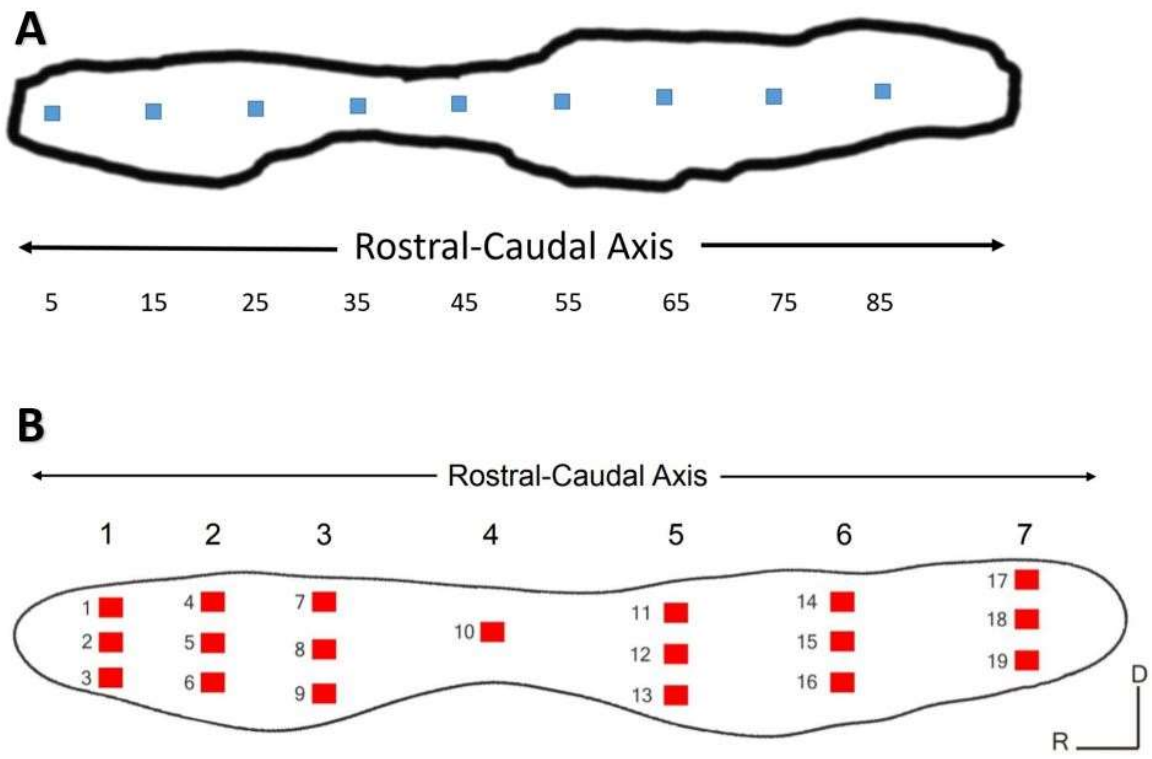

Figure 15. A) Saccule regions for hair cell counting for bigheaded carps in this study. B) Saccule regions used for quantifying hair cell density in *C. auratus* (modified from Smith et al., 2011).

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