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Hearing Sensitivity and the Effect of Sound Exposure on the Axolotl (Ambystoma Mexicanum)

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HEARING SENSITIVITY AND THE EFFECT OF SOUND EXPOSURE ON THE AXOLOTL (*AMBYSTOMA MEXICANUM*)

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HEARING SENSITIVITY AND THE EFFECT OF SOUND EXPOSURE ON THE AXOLOTL (*AMBYSTOMA MEXICANUM*)

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I dedicate this thesis to my parents, Paul and Debbie Fehrenbach. Your love and support have made all of this possible, and I could not have done it without you. Thank you for everything.

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The axolotl (*Ambystoma mexicanum*) has been used as a model organism for studying development, genetics, and regeneration. Although the sensory hair cells of the lateral line of this species have been shown to be able to regenerate, it is not known whether this also occurs in the inner ear. In fact, little is known about the hearing capabilities of the axolotl or other salamander species. I recorded auditory evoked potentials (AEPs) of six axolotls at eleven frequencies (0.1, 0.25, 0.4, 0.6, 0.8, 1, 1.5, 2, 3, 4, and 6 kHz) in order to produce baseline audiograms of underwater pressure sensitivity. Individuals were then subjected to a 48-hour, 150 Hz sound exposure at approximately 170 dB (re 1 µPa). AEPs were then performed to measure hearing thresholds immediately after sound exposure and at 2, 4, and 8 days post-sound exposure (DPSE).

In the baseline audiogram, axolotls were most sensitive at 600 Hz, with an additional peak of sensitivity at 3 kHz. Following sound exposure, axolotls experienced a 6 to 12 dB temporary threshold shift (TTS) after sound exposure, with TTS being greatest at low frequencies near the 150 Hz stimulus frequency (i.e., 100 and 250 Hz). Hearing sensitivity returned to control levels within 8 DPSE. This indicates that axolotls do possess the ability to recover hearing sensitivity after damage following acoustical

trauma. This study is the first to document hearing loss in the axolotl. Future studies are needed to correlate this hearing loss and recovery to sensory hair cell loss and regeneration in the axolotl inner ear.

INTRODUCTION

Salamanders are an excellent biological model for studying the processes of development and regeneration. Stone (1933, 1937) was the first to document limb and tail regeneration in salamanders, while more recent studies show that the sensory cells of the lateral line system are also able to regenerate (Balak et al. 1990). The axolotl (*Ambystoma mexicanum*) is a species of mole salamander that exhibits paedomorphosis, remaining in its aquatic larval form its entire life, making it a particularly interesting model for sensory development and regeneration (Shaffer 1993).

While the axolotl continues to show potential as a model organism for various disciplines, its status in the wild is not as positive. The range of this species is now limited to only two highly-managed bodies of water in the Mexican High Plateau, making the axolotl critically endangered in the wild. The water source of these aquatic habitats is no longer natural – the supply comes from treatment plants. Additionally, these habitats contain up to 10 introduced fish species, some of which act as predators to the axolotl at various life stages (Alcocer-Durand and Escobar-Briones 1992, Zambrano et al. 2007). The pressures of this complex aquatic habitat require the use of various sensory systems. Axolotls can elucidate information about their environment through both olfaction and vision, but little is known about how well they perceive and interpret vibrational stimuli using the sensory cells of the inner ear and lateral line (Eisthen et al. 1994, Deutschlander 1995).

The lateral line system of the skin of fish and aquatic amphibians contain mechanoreceptive sensory organs known as neuromasts, which detect movement and vibration in the surrounding water (Lombard 1980, Lewis and Narins 1999, Bleckmann

and Zelick 2009). Surface neuromasts are composed of sensory hair cells that are covered by a gelatinous cupula which couples them to the surrounding water. These sensory organs are vital to vestibular perception and detecting disturbances in the aquatic environment, particularly vibrational movement of the water (Popper and Fay 1999). A keen awareness of vibrations in the surrounding water is important for both predator detection and prey capture for aquatic species (Coombs and Montgomery 1999, Coffin et al. 2013). When lateral line neuromasts are lost following damage, their sensory hair cells can regenerate in both fishes and aquatic amphibians, including axolotls (Balak et al. 1990, Northcutt et al. 1994).

As hair cells in the teleost lateral line are morphologically, physiologically, and functionally similar to those found in the inner ear (Monroe et al. 2015), and since auditory hair cells have been shown to regenerate in numerous other non-mammalian vertebrates (e.g, fishes, Smith et al. 2006; urodele amphibians, Taylor and Forge 2005; lizards, Avallone et al. 2003; birds, Corwin and Cotanche 1988), it is likely that the sensory hair cells of the inner ear of axolotls are also able to regenerate. This has never been examined in axolotls. In fact, little is known about their hearing ability in general.

The goal of this study was two-fold: to characterize axolotl hearing sensitivity within a frequency range of 100 Hz to 6000 Hz, as well as to examine the effects of sound exposure and recovery on their hearing abilities to ascertain whether the axolotl could be a potential model to investigate inner ear hair cell regeneration. In accordance with previous studies of non-mammalian vertebrates, I predicted that sound exposure would produce hearing loss and that the complete recovery of hearing capabilities in the axolotl would occur after sound exposure.

MATERIALS AND METHODS

Axolotls were obtained from the Ambystoma Genetic Stock Center (Lexington, KY, USA) and maintained individually in 9.5 L flow-through tanks. Mean (± S.E.) total length, snout-vent length, and mass of the axolotls (N=6) were 19.9 (± 0.6) cm, 10.2 (± 0.2) cm, and 59.0 (± 2.5) g, respectively. Electrophysiological hearing tests were performed using a modified version of the current Auditory Evoked Potential (AEP, Table 1) method currently employed for studying the hearing of fishes (Smith et al. 2006, Smith et al. 2011, Uribe et al. 2013, Ladich and Fay 2013). Axolotls were anesthetized with tricaine methanesulfonate (MS-222) at a concentration of 2 g/L. Anesthetized individuals were then suspended using a thin, flexible, plastic mesh in a 19-L tank in order to generate baseline audiograms. The mesh around each individual was attached to an overhead clip (Fig. 1), keeping it 6 cm below the surface of the water and 22 cm above a UW-30 underwater speaker (Electro-Voice, Burnsville, MN). Electrical interference was minimized by keeping the tank within a Faraday cage. This cage was located within a sound-attenuation room to reduce background noise (WhisperRoom, Inc., Knoxville, TN). Three stainless steel sub-dermal electrodes (27 gauge; Rochester Electro-Medical, Inc., Tampa, FL) were used to obtain physiological responses: a ground electrode in the tail muscle, a reference electrode on the tip of the nose, and a recording electrode centrally over the brainstem, approximately 1 cm posterior to the eyes (Fig. 1).

BioSig software (Tucker Davis Technologies, Alachua, FL) was used to generate pure tone stimuli at eleven different frequencies (0.1, 0.25, 0.4, 0.6, 0.8, 1, 1.5, 2, 3, 4, and 6 kHz). All tones were played first at a phase of 90° and then again at 270° in order

to cancel out any electrical signal emitted by the underwater speaker that could potentially interfere with the AEP traces. Each frequency was tested by decreasing the sound pressure level in 5 dB steps until an AEP trace was no longer visible. The lowest sound pressure level at which an AEP waveform was visible was noted as the threshold for each frequency. The collective thresholds for these eleven frequencies were used to produce audiograms.

After control levels were established, axolotls were experimentally sound-exposed for 48 hours. The sound exposure maintained a constant 150 Hz tone at a mean sound pressure level throughout the 19-L tank of 170 dB (re 1 µPa). This was determined by characterizing sound pressure for a number of depths within the 22.5-cm deep exposure setup. The sound levels ranged from 161.5 near the water's surface to 174.7 dB (re 1 µPa) 1 cm from the speaker. Axolotls were allowed to swim freely throughout the sound exposure tank. After deafening, AEP hearing tests were performed 0, 2, 4, and 8 days post-sound exposure (DPSE) using the same procedures for control audiograms. All procedures were conducted under the approval of the Western Kentucky University Institutional Animal Care and Use Committee (Animal Welfare Assurance # A3558-01). In order to compare control level hearing thresholds of the baseline audiogram to those of the audiograms generated for 0, 2, 4, and 8 DPSE, overall paired t-tests (averaged across all frequencies) were performed for each experimental day. Additional paired t-tests were performed to compare thresholds for each frequency. P-values were adjusted for multiple comparisons using Holm's sequential Bonferroni method (Abdi 2010, Eichstaedt et al. 2013). Paired t-tests were only performed for comparisons where standard error bars did not overlap in order to retain statistical power.

Additionally, two-way ANOVAs were performed to address frequency and day effects on TTS. TTS was calculated as the post-sound exposure threshold minus the mean control (pre-sound exposure) threshold. Finally, a linear regression was performed to examine the relationship between TTS and DPSE. All tests were performed at the alpha = 0.05 level. SYSTAT 13 software (SYSTAT Software, Inc., San Jose, CA) was used for all statistical analyses.

RESULTS

Axolotls detected the sound stimuli at all frequencies examined from 0.1 to 6 kHz. The baseline axolotl audiogram (control) exhibited two peaks of sensitivity, at 0.6 and 3 kHz, where their thresholds were 118 and 123 dB re 1 µPa, respectively (Fig. 2). Hearing thresholds were tested for differences associated with sex, mass, total length, and snout-to-vent length and no significant differences were found due to sex or size. Initial overall paired t-tests (averaged across all frequencies) showed that there was a significant difference between the control and 0 DPSE thresholds ($t = 2.01$, $df = 5$, $p = 0.036$). This departure from control levels indicates that the sound exposure significantly damaged the hearing ability of the axolotls (i.e. caused a temporary threshold shift). There was also a significant effect of sound exposure at 2 DPSE ($t = 2.01$, $df = 5$, $p = 0.001$). By 4 and 8 DPSE, there was no longer a significant overall difference between control and experimental audiograms, but at 4 DPSE there was a significant interaction between treatment and frequency, meaning that sound-exposed axolotls only had higher thresholds than controls at specific frequencies.

In addition to overall paired t-tests, additional paired t-tests were performed to examine treatment differences by individual frequency. Hearing thresholds were significantly different from control levels for 0.1, 0.8, and 2 kHz at 0 DPSE, and at 0.1, 1, and 2 kHz for 2 DPSE (Fig. 2, Table 2). While there was no significant overall difference between control and sound-exposed thresholds at 4 DPSE and 8 DPSE, significant differences were exhibited at 0.1 kHz at 4 DPSE.

Separate ANOVAs were performed to test for frequency and day effects on TTS. Analyses were performed using frequencies less than or equal to 600 Hz as these frequencies were most strongly affected by the low frequency of the experimental sound exposure. TTS significantly differed with frequency at 2 DPSE ($F_{3,20} = 7.22$, $p=0.002$), 4 DPSE ($F_{3,20} = 43.37$, $p<0.001$), and 8 DPSE ($F_{3,20} = 3.16$, $p=0.047$). As a 150 Hz tone was used for the sound stimulus, TTS was greatest at the lower frequencies tested (100 and 250 Hz; Fig. 3). TTS also significantly differed by day for each frequency, with TTS being greatest immediately after sound exposure and least at 8 DPSE: 100 Hz ($F_{3,20} = 10.03$, $p<0.001$), 250 Hz ($F_{3,18} = 5.97$, $p=0.005$), 400 Hz ($F_{3,20} = 5.41$), and 600 Hz ($F_{3,20} = 3.79$, $p=0.027$). TTS decreased linearly with time following sound exposure, with thresholds returning to control levels by 8 DPSE (Fig. 4).

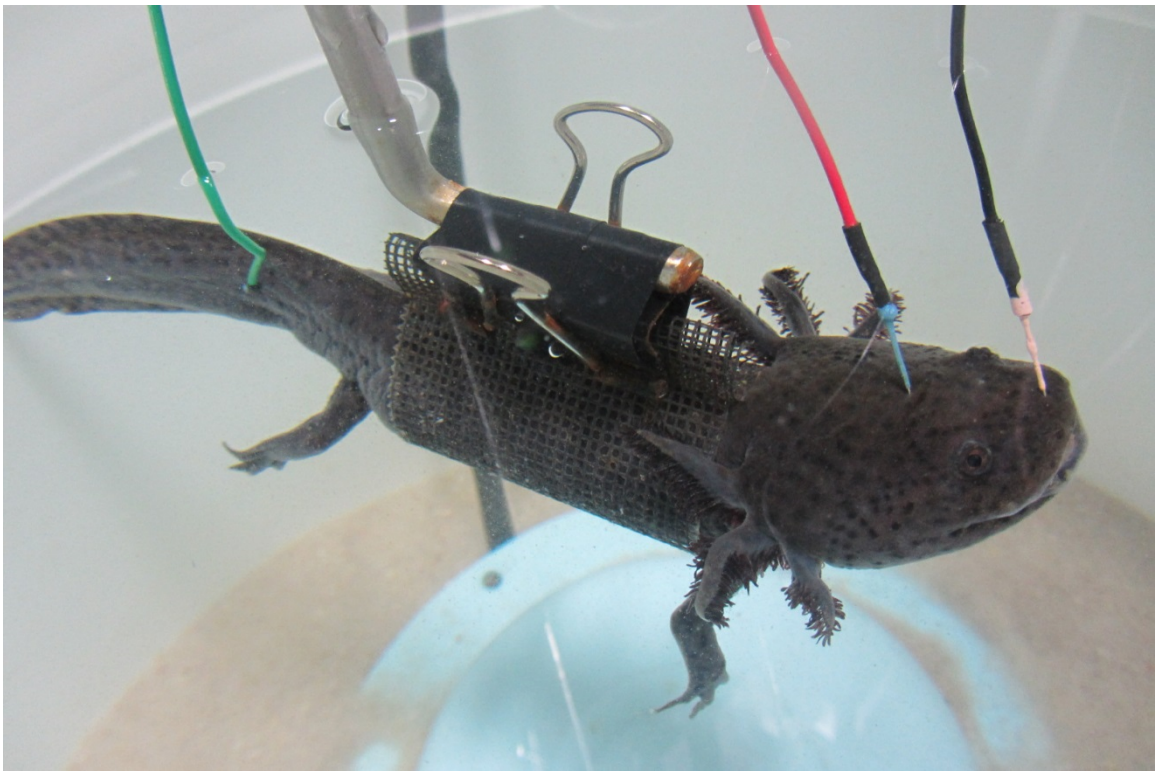

Figure 1 – Electrode placement in the axolotl auditory evoked potential (AEP) setup. The red, black, and green electrodes are recording, reference, and ground electrodes, respectively. The UW-30 underwater speaker (light blue) is shown below the axolotl.

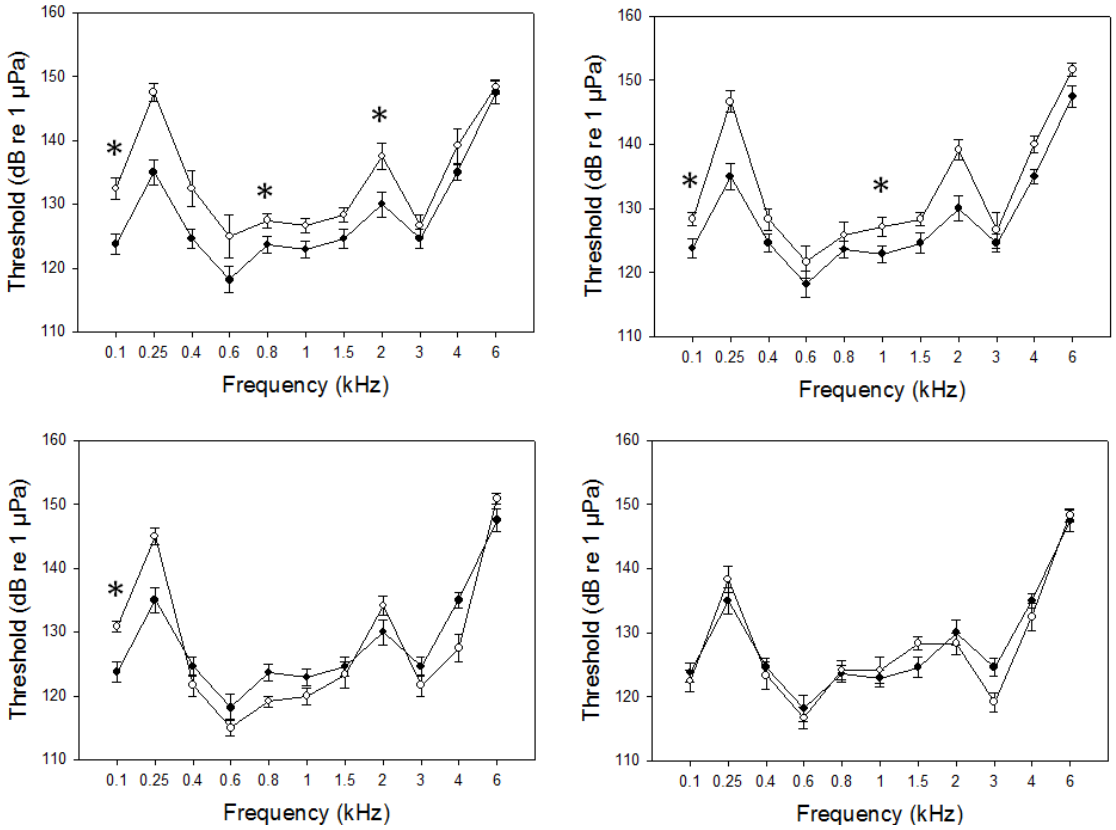

Figure 2 – Four graphs showing mean (± S.E.) control (filled circles) and the experimental (open circles) thresholds for 0, 2, 4, and 8 days post-sound exposure (DPSE). Asterisks mark significant differences between treatment and control thresholds using Holm's sequential Bonferroni-corrected p-values at the alpha level of 0.05 (see Table 2).

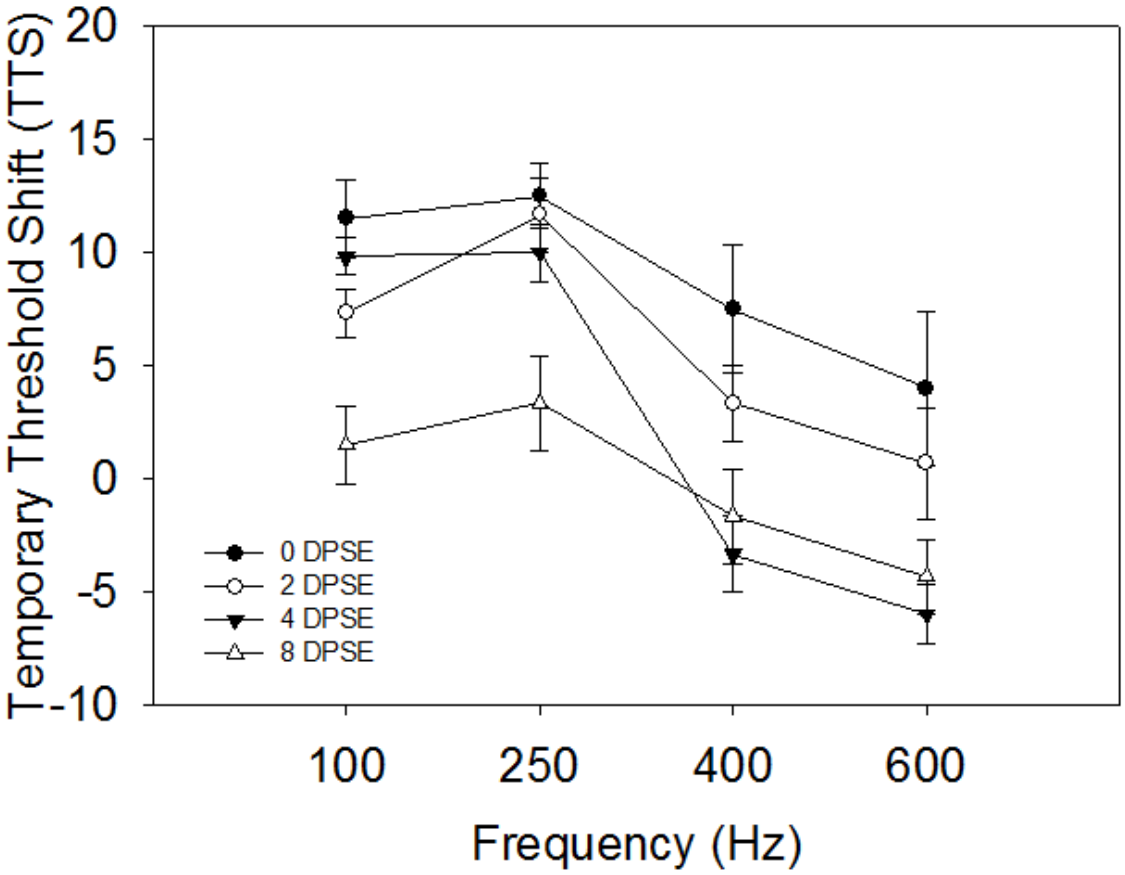

Figure 3 – Mean (±SE) temporary threshold shift (TTS) as a function of test frequency and day post-sound exposure (DPSE).

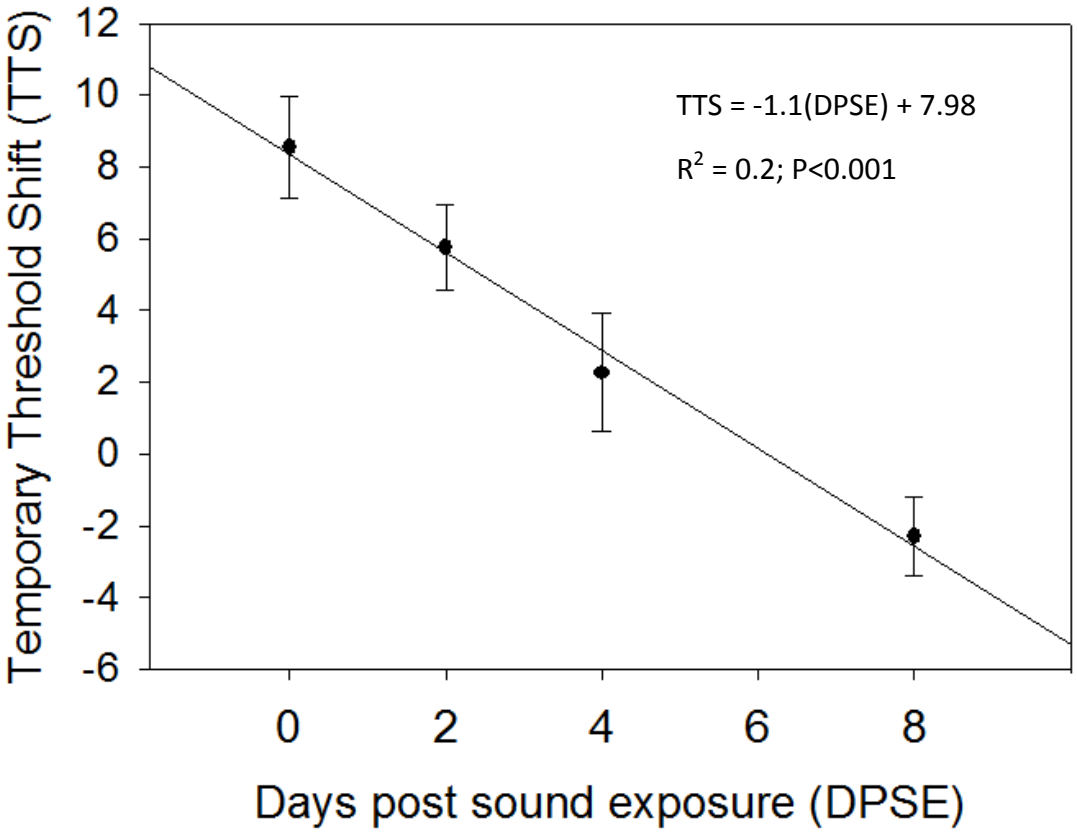

Figure 4 – Mean (± S.E.) temporary threshold shift (TTS) as a function of days post-sound exposure (DPSE). Regression analysis shows a significant negative linear relationship between the two variables, indicating improvement of hearing over time.

Table 1 – Alphabetically-ordered abbreviations from the text with corresponding definitions.

Abbreviation	Definition
AEP	Auditory Evoked Potential
AP	Amphibian Papilla
DPSE	Days Post Sound Exposure
MS-222	Tricaine Methanesulfonate
TTS	Temporary Threshold Shift

Table 2 – Holm's sequential Bonferroni-corrected p-values for all frequencies at each Day Post-Sound Exposure (DPSE). Comparisons that show significance at the alpha level of 0.05 are bolded. Dashes indicate comparison was not made due to overlapping error bars.

Frequency	0 DPSE	2 DPSE	4 DPSE	8 DPSE
100	**0.0026**	**0.0444**	**0.0016**	--
250	0.4439	0.0547	0.1657	--
400	0.1669	0.1648	--	--
600	0.2589	--	--	--
800	**0.0058**	--	0.2620	--
1000	0.1226	0.0469	0.3632	--
1500	0.5448	0.2325	--	0.1816
2000	**0.0361**	**0.0497**	0.2103	--
3000	--	--	--	0.2242
4000	0.3739	0.1780	0.1410	--
6000	--	0.2420	--	--

DISCUSSION

Axolotl baseline hearing

In general, there is little information known about hearing in aquatic salamanders, although hearing ability has been examined in some terrestrial salamanders as well as other species of amphibians. For example, behaviorally-determined audiograms have been generated for the olm (*Proteus anguinus*), a blind cave salamander, as sound is a vital stimulus to a species without a sense of sight. Sound was detected between a frequency range of 10 Hz and 10 kHz, with peak sensitivities occurring at approximately 2 kHz and 10 kHz (Bulog and Schlegel 2000). Previous researchers have used a more proximate approach by applying vibration directly to the oval window of the inner ear (Hilton 1949). A vibrational probe was used to directly stimulate the inner ear of the Eastern newt (*Notophthalmus viridescens*), and subsequent eighth cranial nerve responses were recorded. The results showed an overall peak frequency of sensitivity at 0.4 kHz, although 11 of the 28 recordings displayed W-shaped vibrational audiograms, meaning they showed two peaks of sensitivity (Ross and Smith 1982). Hearing tests performed on adult tiger salamanders from a 0.1 to 1 kHz range also showed a two-peak trend, with lower frequency sensitivity attributed to particle motion detection and higher frequency sensitivity attributed to pressure detection (Hetherington and Lombard 1983). All of these findings show similarities to my resulting baseline audiogram, as my data reveal that axolotls can detect pressure from at least 0.1 to 6 kHz, with two peaks of sensitivity at both 0.6 and 3 kHz. Lastly, recent hearing tests performed in air on the axolotl display a W-shape with two peaks of sensitivity, for both vibrational/particle motion stimuli (shaker table) exposure and pressure (loudspeaker) exposure. This novel exploration into

aerial axolotl audition shows that this species can detect aerial sound in the frequency range of at least 0.2 to 1.3 kHz, with peak sensitivities occurring at both 80 and 240 Hz for particle motion and 80 and 320 Hz for pressure (Christensen et al. 2015a).

I hypothesize that the two peaks of sensitivity are related to the axolotl's phylogenetic position in relation to the evolution of hearing. In general, fishes tend to hear best at frequencies lower than 1 kHz (Fay 1988). These sensitivity peaks could correspond to those of both ancestral fish relatives (as lower frequency sounds are more prevalent and travel farther in aquatic environments) as well as terrestrial tetrapod relatives (as higher frequency stimuli are often more important in terrestrial environments). The axolotl does not spend any part of its life cycle outside of the aquatic environment, and therefore does not possess any anatomical adaptations for terrestrial hearing, such as a tympanum. The axolotl shares this characteristic with the first terrestrial vertebrates, who did not develop a tympanic middle ear until approximately 100 MY after their appearance on land (Christensen et al. 2015a). This suggests that salamanders are no more suited to hearing on land than fishes would be. However, amphibians do possess unique hearing adaptations such as the amphibian papilla (AP), which is a specialized auditory sensory organ thought to be responsible for the bulk of amphibian acoustic sensitivity (Lewis and Narins 1999).

The anatomy of the AP suggests that it is responsible for detecting substrate vibrations and fluid displacement, a function analogous to that of the lateral line (Smith 1968). Direct vibrational stimulus to the oval window of one ear causes a cascade of vibration that passes through both the right and left AP in salamanders, classifying their hearing as binaural (Wever 1978). Amphibians also have a columella bone which is an

adaptation for conducting acoustic energy to the ear. In the axolotl, the columella bone is free in both the juvenile and adult life stages, but fused to the bony labyrinth known as otic capsule in the terrestrial tiger salamander (*Ambystoma tigrinum*). The fusion to the otic capsule surrounding the inner ear suggests that vibrations affecting the columella are directly imparted to the inner ear, enhancing acoustic detection in the tiger salamander, but not the axolotl (Christensen et al. 2015a). It has also been long known that amphibians such as salamanders, frogs, and toads are sensitive to seismic substrate vibrations (Ross and Smith 1978, Narins 1990). Anatomical and physiological studies show that this sensitivity is largely due to the sacculus of the inner ear and not the AP (Lewis et al. 1982).

Another hearing adaptation of aquatic vertebrates is the use of enclosed volumes of air to amplify sound energy, such as the swim bladders of fish. Some fish possess Weberian ossicles, which are derived vertebrae used to amplify sound pressure stimuli by connecting the inner ear to the swim bladder (Fay and Popper 1980, Popper and Fay 1999). The use of air volumes mediating salamander hearing has been examined in the tiger salamander (*Ambystoma tigrinum*), a close relative to the axolotl (Hetherington and Lombard 1983). This study found that when the mouth cavity was filled with air, sound pressure was transduced as vibrations that were transmitted to the inner ear, likely due to the proximity of the inner ear to the mouth. Another potential mode of pressure transduction for salamanders is via air volumes in lung cavities.

Recent work by Christensen et al. (2015a) measured air volumes in both the mouth and lungs of the axolotl, examined their hearing in water across six frequencies ranging from 80 Hz to 640 Hz, and also assessed their hearing ability in air. CT scans of

axolotls did not show any air in the mouth cavity of conscious specimens, and negligible volumes in anaesthetized individuals. This indicates that there is no evidence for the use an air-filled mouth cavity to transduce pressure stimuli in this species. However, the lungs of both juvenile and adult axolotls contained 2–3 ml air, which corresponds to a resonance frequency of 0.4 to 0.5 kHz. This finding suggests that axolotls may be able use air in the lung cavity to transduce sound pressure into particle motion in the inner ear, a phenomenon also noted in lungfish (Christensen 2015b). It should be noted, however, that this study did not find any distinct anatomical connection between the lung cavity and the inner ear. Christensen et al. (2015a) also examined particle motion versus pressure detection in water across the previously mentioned frequencies. The six frequencies tested in this study partially overlap the frequency range used in the current study, showing similar trends in audiogram shape across frequencies. The results of the aquatic particle motion and pressure sensitivity tests showed that axolotls detect particle motion as the primary stimulus at low frequencies and pressure at those above 120 Hz.

However, it is possible that some of the detection of low frequency particle motion found by Chistensen et al. (2015a), as well as the low frequency sensitivity in my results, could be attributed to the lateral line sensory cells, as they are specialized for low frequency detection (Coombs and Montgomery 1999, Coffin et al. 2013). The underwater sound pressure sensitivities of axolotls reported by both Christensen et al. (2015a) and my current work exhibit an upside-down U-shape at lower frequencies, with thresholds being lower at 80 or 100 Hz, increasing for intermediate frequencies, and then decreasing again at 640 or 700 Hz. The increased sensitivity at 100 Hz shown in my generated baseline audiogram could be ascribed to the low frequency sensitivity of the

lateral line system. In order to generate an audiogram based solely on inner-ear sensitivity, the AEP procedure should be repeated following chemical or mechanical ablation of the lateral line (Higgs and Radford 2013).

Effects of sound exposure and recovery

This study is the first example of experimental hearing loss in a salamander species. Here I show that the axolotl auditory system is susceptible to damage from intense sounds like that of other vertebrates, and like other non-mammalian vertebrates, can recover from some acoustic trauma to the auditory system within a matter of days. Although not measured directly in this study, it is presumed that the hearing loss was due to damage to the auditory hair cells of the inner ear. This hypothesis will need to be confirmed in future axolotl studies, but previous studies of hearing loss in fishes show a significant correlation between inner ear hair cell loss and hearing loss (Hastings et al. 1996, Smith et al. 2004, Schuck and Smith 2009, Smith et al. 2011, Monroe et al. 2015).

Fishes exposed to loud sounds exhibit sensory hair cell damage and temporary threshold shifts (TTS) in some species, with hearing recovering as hair cells regenerate (Lombarte 1993, Smith 2006). For example, regeneration of the inner ear sensory hair cells of goldfish (*Carassius auratus*) return to baseline levels within 14 days following intense noise exposure for 21 days (Smith et al. 2004). However, fishes are not the only organisms with this capability. Sensory hair cell regeneration has been found in every non-mammalian vertebrate examined so far, including salamanders (Balak et al. 1990, Avallone et al. 2008, Brignull et al. 2009, Monroe et al. 2015).

The lateral line system of the axolotl has been shown to regenerate following laser ablation or fluorescent excitation, with the first new hair cells appearing 3-5 days after neuromast ablation (Balak et al. 1990). It was concluded that mature sensory epithelia that have been completely depleted of hair cells can still generate new hair cells, and that preexisting hair cells are not necessary for regeneration. The striking similarity of lateral line hair cells to inner ear hair cells (Popper and Fay 1999), suggests that amphibians should be able to regenerate auditory hair cells as well. This, indeed, is the case. The American bullfrog (*Lithobates catesbeianus*) can recover lost hair cells in the AP after noise-induced damage (Simmons et al. 2014). Recovery from hair cell damage occurred over a time course of nine days, which is comparable to the 8-day recovery that was observed in axolotls.

Recovery rates have been shown to vary between the sensory cells of the lateral line system and those of the inner ear in fish species. In zebrafish (*Danio rerio*), neuromasts of the lateral line system are able to regenerate back to control levels after about 72 hours following chemical ablation with a large proportion of cell proliferation occurring in the first day post-damage (Hernandez 2007, Coffin et al. 2013). In contrast, inner ear hair cell bundles in the zebrafish require more time to fully regenerate following damage, taking approximately 14 days to return to control levels following acoustical trauma (Smith et al. 2011). If this disparity between the timing of sensory hair cell regeneration of the lateral line and inner ear is also found in the axolotl, it could have affected the results of this study if any of the lateral line sensory cells were damaged during the 48-hour sound exposure. If this is the case, the 8-day time course for hearing recovery found in this experiment envelopes a shorter lateral line recovery within that 8-

day period. As mentioned previously, in order to assess the importance of the lateral line sensory cells in both the baseline audiogram of this species as well as its role in hearing recovery, this study should be replicated following ablation of the lateral line neuromasts.

In response to the low frequency (150 Hz) stimulus used in this experiment to induce hearing loss, threshold shifts were greatest at the two lowest frequencies tested (100 and 250 Hz; Fig. 3). This is likely because the inner ear sensory epithelia of the axolotl are tonotopically organized. That is, there is an orderly arrangement of frequency response in the auditory sensory organs. Although this has not been specifically studied in any salamander species, it has in other aquatic or semi-aquatic taxa such as fishes and anuran amphibians. For example, the goldfish (*Carasius auratus*) saccule is tonotopically organized, with intense, low frequency sounds damaging hair cells in the caudal portion, while high frequency sounds affect the rostral saccule (Smith et al. 2011). Frogs have a three-part auditory system that detects low (the sacculus), mid (the AP), and high (the basilar papilla) frequencies (Smotherman and Narins 2000). At a finer scale of analysis, the AP is further tonotopically organized. The AP of the northern leopard frog (*Rana pipiens pipiens*) exhibit three morphologically distinct regions that that differ in hair cell length and shape, which correlates to tonotopic sensitivity of the AP (Simmons et al. 1994).

Due to their susceptibility to hearing loss after sound exposure and then subsequent recovery, axolotls may make a good model organism for examining the process of inner ear hair cell regeneration. Zebrafish are currently a popular model organism for such studies (Schuck and Smith 2009, Sun et al. 2011), but the use of salamanders such as axolotls has some distinct advantages. First, the axolotl requires low

maintenance in captivity, similar to that of the zebrafish, making it easy to keep and propagate in a laboratory setting. Second, the axolotl's popularity in genetic, developmental, and gerontological studies has led to the design of many different genetic strains and mutants of this species, including various color morphs which may prove useful to hearing research investigating oto-protective pigmentation (Shaffer 1993, Roy and Gatien 2008, Coffey 2014). Third, although there are various model organisms with some capacity for tissue regeneration (such as mouse and chicken embryos, Drosophila imaginal discs, and zebrafish), only a salamander such as an axolotl has been shown to regenerate entire tails, limbs, and even spinal cords (Clarke et al. 1988). The regeneration of so many different tissue types in one organism yields deeper insight into the application of these findings for humans and other organisms that do not possess that ability (Roy and Gatien 2008, McCusker and Gardiner 2011). Finally, specific to auditory regeneration, axolotls have a larger inner ear epithelium, potentially easing the difficulties of dissection and culturing of sensory epithelium associated with a model as small as the zebrafish. Future studies addressing inner ear hair cell densities, tonotopy, and sensory hair cell regeneration in the axolotl inner ear will be needed to develop the axolotl as a biomedical model for the hearing sciences.

LITERATURE CITED

Abdi H. 2010 Holm's sequential Bonferroni procedure. In *Encyclopedia of Research Design*, pp. 1-8. Thousand Oaks, CA: SAGE Publications, Inc.

Alcocer-Durand J, Escobar-Briones EG. 1992 The aquatic biota of the now extinct lacustrine complex of the Mexico Basin. *Freshw. Forum* **2**, 1-13.

Avallone B, Porritiello M, Esposito D, Mutone R, Balsamo G, Marmo F. 2003 Evidence for hair cell regeneration in the crista ampullaris of the lizard *Podarcis sicula*. *Hearing Res.* **178**, 79-88.

Avallone B, Fascio U, Balsamo G, Marino F. Gentamicin ototoxicity in the saccule of the lizard *Podarcis sicula* induces hair cell recovery and regeneration. *Hearing Res.* **235**, 15-22.

Balak KJ, Corwin JT, Jones JE. 1990 Regenerated hair cells can originate from supporting cell progeny: evidence from phototoxicity and laser ablation experiments in the lateral line system. *J. Neurosci.* **10**, 2502-2512.

Bleckmann H, Zelick R. 2009 Lateral line system of fish. *Integr. Zool.* **4**, 13-25.

Brignull HR, Raible DW, Stone JS. 2009 Feathers and fins: non-mammalian models for hair cell regeneration. *Brain Res.* **1277**, 12-23.

Bulog B, Schegel P. 2000 Functional morphology of the inner ear and underwater audiogram of *Profeus anguinus* (Amphibia, Urodela). *J. Physiol.* **439**, 165-167.

Christensen CB, Lauridsen H, Christensen-Dalsgaard J, Pedersen M, Madsen PT. 2015a Better than fish on land? Hearing across metamorphosis in salamanders. *Proc. R. Soc. B* **282**, 20141943.

Christensen CB, Christensen-Dalsgaard J, Telberg Madsen P. 2015b Hearing of the African lungfish (*Protopterus annectens*) suggests underwater pressure detection and rudimentary aerial hearing in early tetrapods. *J. Exp. Biol.* **218**, 381-387.

Clarke JD, Alexander R, Holder N. 1988 Regeneration of descending axons in the spinal cord of the axolotl. *Neurosci. Lett.* **1**, 1-6.

Coffey BN. 2014 Melanin as an oto-protective pigment in two fish species: *Poecilia latipinna* and *Cyprinus carpio*. Master's Thesis, Western Kentucky University, 46 pp.

Coffin AB, Brignull H, Raible DW, Rubel EW. 2013 Hearing loss, protection, and regeneration in the larval zebrafish lateral line. In *The Lateral Line System,* pp. 322-344. New York, NY: Springer-Verlag, Inc.

Coombs S, Montgomery JC. 1999 The enigmatic lateral line system. In *Comparative Hearing: Fish and Amphibians*, pp. 319-352. New York, NY: Springer-Verlag, Inc.

Corwin JT, Cotanche DA. 1988 Regeneration of sensory hair cells after acoustic trauma. *Science* **240**, 1772-1774.

Deutschlander ME. 1995 Characterization of an ultraviolet photoreception mechanism in the retina of an amphibian, the axolotl (*Ambystoma mexicanum*). *Neurosci. Lett.* **2**, 93-96.

Eichstaedt KE, Kovatch K, Moroof DA. 2013. A less conservative method to adjust for familywise error rate in neuropsychological research: the Holm's sequential Bonferroni procedure. *NeuroRehabiliation* **32**, 693-696.

Eisthen HL, Sengelaub DR, Schroeder DM, Alberts JR. 1994 Anatomy and forebrain projections of the olfactory and vomeronasal organs in axolotls (*Ambystoma mexicanum*). *Brain Behav. Ecol.* **2**, 108-124.

Fay RR. 1988 *Hearing in Vertebrates: a Psychophysics Databook*. Winnetka, IL:Hill-Fay Associates.

Fay RR, Popper AN. 1980 Structure and function in the teleost auditory systems. In *Comparative Studies of Hearing in Vertebrates*, pp. 3-40. New York, NY: Springer-Verlag, Inc.

Hastings MC, Popper, AN, Finneran JJ, Lanford PJ. 1996 Effects of low-frequency underwater sound on hair cells of the inner ear and lateral line of the teleost fish *Astronotus ocellatus*. *J. Acoust. Soc. Am.* **99**, 1759-1766.

Hernandez PP, Olivari FA, Sarrazin AF, Sandoval PC, Allende ML. 2007 Regeneration in zebrafish lateral line neuromasts: expression of the neural progenitor cell marker Sox2 and proliferation-dependent and –independent mechanisms of hair cell renewal. *Dev. Neurobiol.* **67**, 637-654

Hetherington TE, Lombard RE. 1983 Mechanisms of underwater hearing in larval and adult tiger salamanders *Ambystoma tigrinum*. *Comp. Biochem. Physiol.* **74A**, 555-559.

Higgs DM, Radford CA. 2013 The contribution of the lateral line to 'hearing' in fish. *J. Exp. Biol.* **216**, 1484-1490

Hilton WA. 1949 The sound-transmitting apparatus of salamanders. *Herpetologica*. **5**, 33-43.

Ladich F, Fay RR. 2013 Auditory evoked potential audiometry in fish. *Rev. Fish. Biol.*

Fisher. **23**, 317-364.

Lewis ER, Baird RA, Leverenz EL, and Koyama H. 1982 Inner ear: injection reveals peripheral origins of specific sensitivities. *Science* **215**, 1641-1643.

Lewis ER, Narins PM. 1999 The acoustic periphery of amphibians. *In Comparitive Hearing: Fish and Amphibians*, pp. 101-141. New York, NY: Springer-Verlag, Inc.

Lombard RE. 1980 The structure of the amphibian auditory periphery: a unique experiment in terrestrial hearing. In *Comparative Studies of Hearing in Vertebrates*, pp. 121-161. New York, NY: Springer-Verlag, Inc.

Lombarte A, Yan H, Popper AN, Chang J, Platt C. 1993 Damage and regeneration of hair cell ciliary bundles in a fish ear following treatment with gentamicin. *Hearing Res.* **64**, 166–174.

McCusker C, Gardiner DM. 2011 The axolotl model for regeneration and aging research: a mini-review. *Gerontology* **57**, 565-571.

Monroe JD, Rajadinakaran G, Smith ME. 2015 Sensory hair cell death and regeneration in fishes. *Front. Cell. Neurosci.* **9**, 131.

Narins PM. 1990 Seismic communications in anuran amphibians. *Bioscience* **40**, 268-274.

Northcutt RG, Catania KC, and Criley BB. 1994 Development of lateral line organs in the axolotl. *J. Comp. Neurol.* **340**, 480–514.

Popper AN, Fay RR. 1999 The auditory periphery in fishes. In *Comparitive Hearing: Fish and Amphibians*, pp. 43-98. New York, NY: Springer-Verlag, Inc.

Ross RJ, Smith JJB. 1978 Detection of substrate vibrations by salamanders: inner ear sense organ activity. *Can. J. Zool.* 6**6**, 1156-1162.

Ross RJ, Smith JJB. 1982 Responses of the salamander inner ear to vibrations of the middle ear. *Can. J. Zool.* **60**, 220-226.

Roy S, Gatien S. 2008 Regeneration in axolotls: a model to aim for! *Exp. Gerontol.* **43**, 968-973.

Schuck JB, Smith ME. 2009 Cell proliferation follows acoustically-induced hair cell bundle loss in the zebrafish saccule. *Hearing Res.* **253**, 67-76.

Shaffer HB. 1993 Phylogenetics of model organisms: the laboratory axolotl, *Ambystoma mexicanum*. *Syst. Biol.* **42**, 508-522.

Simmons DD. 2014 Recovery of otoacoustic emissions after high-level noise exposure in the American Bullfrog. *J. Exp. Biol.* **217**, 1626-2636.

Simmons DD, Bertolotto C, Narins PM. 1994 Morphological gradients in sensory hair cells of the amphibian papilla of the frog, *Rana pipiens pipiens*. *Hearing Res*. **80**, 71-78.

Smith ME, Kane AS, Popper AN. 2004 Noise-induced stress response and hearing loss in goldfish (*Carassius auratus*). *J. Exp. Biol.* **207**, 427-435.

Smith ME, Coffin AB, Miller DL, Popper AN. 2006 Anatomical and functional recovery of the goldfish (*Carassius auratus*) ear following noise exposure. *J. Exp. Biol.* **209**, 4193-4202.

Smith ME, Schuck JB, Gilley RR, Rogers BD. 2011 Structural and functional effects of acoustic exposure in goldfish: evidence for tonotopy in the teleost saccule. *BMC Neurosci.* **12**, 19.

Smith JJB. 1968 Hearing in terrestrial urodeles: a vibration-sensitive mechanism in the ear. *J. Exp. Biol.* **48**, 191-205.

Smotherman MS, Narins PM. 2000 Hair cells, hearing and hopping: a field guide to hair cell physiology in the frog. *J. Exp. Biol.* **203**, 2237-2246.

Stone LS. 1933 The development of lateral-line sense organs in amphibians observed in living and vital-stained preparations. *J. Comp. Neurol.* **57**, 507-540.

Stone LS. 1937 Further experimental studies of the development of lateral-line sense organs in amphibians observed in living preparations. *J. Comp. Neurol.* **68**, 83-115.

Sun H, Lin C-H, Smith ME. 2011 Growth hormone promotes hair cell regeneration in the zebrafish (*Danio rerio*) inner ear following acoustic trauma. *PLoS ONE.* **6**, e28372.

Taylor RR, Forge A. 2005 Hair cell regeneration in sensory epithelia from the inner ear of a urodele amphibian. *J. Comp. Neurol*. **484**, 105-120.

Uribe PM, Sun H, Wang K, Asuncion JD, Wang Q, Steyger PS, Smith ME, Matsui JI. 2013 Aminoglycoside-induced hair cell death of inner ear organs causes functional deficits in adult zebrafish (*Danio rerio*). *PLoS ONE* **8**, e58755.

Wever EG. 1978 Sound transmission in the salamander ear. *Proc. Nat. Acad. Sci. USA* **75**, 529-530.

Zambrano L, Vega E, Herrara M, Prado E, Reynoso VH. 2007 A population matrix model and population viability analysis to predict the fate of endangered species in highly managed water systems. *Anim. Conserv.* **10**, 297-303.