DOI: 10.4274/jarem.galenos.2025.29964
J Acad Res Med 2025;15(1):10-16

Objective Evaluation of Auditory Function of Axolotls Pre- and Post-metamorphosis and its Comparison with Rats

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Cite this article as: Büyükkal F, Öztürk ŞT, Şerbetçioğlu MB. Objective evaluation of auditory function of axolotls pre- and post-metamorphosis and its comparison with rats. J Acad Res Med. 2025;15(1):10-16

ABSTRACT

Objective: Axolotls possess remarkable regenerative capabilities, including the ability to regenerate the brain, spinal cord, internal organs, lateral line system cells, and inner ear hair cells. However, following metamorphosis, their regenerative capacity diminishes, accompanied by changes in auditory function. This study aimed to objectively assess these changes to enhance understanding of the relationship between metamorphosis, regeneration, and auditory function, with potential implications for regenerative biology and auditory research. Additionally, 3-month-old rats were included as a comparative model for evaluating auditory function.

Methods: Auditory function in axolotls (maximum length: 20 cm) and 3-month-old rats was evaluated using auditory brainstem response (ABR) testing. ABR measurements were conducted on axolotls both pre- and post-metamorphosis using tone burst stimuli at 250 Hz, 500 Hz, 600 Hz, and 800 Hz. In rats, measurements were taken at 8, 12, 16, and 20 kHz frequencies.

Results: ABR recordings in axolotls pre- and post-metamorphosis revealed that 600 Hz produced the most consistent wave morphology. Wave II latencies were significantly longer before metamorphosis compared to after metamorphosis, indicating alterations in auditory processing. In contrast, 3-month-old rats exhibited stable auditory thresholds across all tested frequencies, demonstrating consistent auditory function.

Conclusion: This study presents the first successful application of ABR methodology for evaluating auditory function in axolotls, providing a comparative analysis with auditory function in 3-month-old rats. Significant changes in auditory function were observed in axolotls following metamorphosis, indicating a decline in auditory capabilities concurrent with the reduction in regenerative capacity. These findings underscore the feasibility of using ABR testing in axolotls and highlight important implications for auditory function research across different species.

Keywords: Ambystoma mexicanum, axolotl hearing, regeneration, rat hearing, animal experimentation

INTRODUCTION

Rats have become a fundamental model in auditory research due to their anatomical and physiological similarities to the human auditory system, as well as their suitability for behavioral and electrophysiological studies. As the second most commonly used laboratory animal after mice, rats have proven essential in understanding auditory processing, hearing loss mechanisms, and neuroplasticity within the auditory pathway (1,2). Their ability to perform complex auditory tasks and adapt to both naturalistic and controlled experimental settings makes them invaluable for investigating sensory processing and auditory cognition (3). The application of advanced imaging techniques, such as functional magnetic resonance imaging and auditory brainstem response (ABR) measurements, has further expanded insights into rat auditory function, enabling researchers to study neural activity patterns linked to auditory perception and disorders (4-6). These approaches have provided significant contributions to our understanding of auditory networks and their relevance to human hearing and auditory-related diseases (5,6).

The use of rats in hearing research has also been instrumental in the development of therapeutic strategies for hearing loss and auditory neuropathies, especially in exploring treatments for noise-induced hearing loss, age-related hearing decline, and ototoxicity (7). The larger brain and auditory structures of rats, compared to those of mice, offer enhanced precision for surgical and imaging techniques, facilitating a more comprehensive investigation of auditory anatomy and neural pathways (8).

In some invertebrates, such as sponges and cephalopods, the potential for regeneration is markedly higher, but it decreases progressively from fish to mammals (9,10). In most mammals, however, the hair cells responsible for hearing, located in the inner ear, have lost their ability to regenerate, resulting in

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Received Date: 04.12.2024 **Accepted Date:** 25.03.2025
Publication Date: 06.05.2025

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permanent hearing loss and disturbances of balance. In contrast, certain organisms, such as the axolotl and the zebrafish, retain the ability to regenerate hair cells throughout their lives (11-14). While mammalian regeneration typically remains at the tissue level, salamanders are more remarkable because they have the ability to regenerate entire organs, a feature that distinguishes them from other amphibians. Salamanders are of great interest in regenerative biology because they can regenerate amputated or severely damaged organs (15).

Among salamanders, Ambystoma mexicanum (commonly known as the axolotl), a species within the family Ambystomatidae, has received considerable attention from regenerative researchers. Studies show that axolotls are able to regenerate not only their amputated limbs, but also their hearts, brains, spinal cords, and several other internal organs (9). Studies in axolotls often focus on the regeneration of the tail. This is because the tail contains a range of tissues, muscle, connective tissue, and nerves, making it an ideal model for studying regeneration.

The axolotl begins to grow limb buds, which later elongate and differentiate into fully functional limbs, after the development of their external gills. As these developmental changes occur, axolotls typically reach a length of 20-28 cm (16). Although the regenerative capacity of axolotls is well known, to our knowledge very few studies have assessed axolotl auditory function in relation to their regenerative capacity. The present study therefore represents the first objective documentation of the auditory function of the salamander.

The tissue impedance of axolotls is higher than that of air. Therefore, a significant amount of incoming sound energy is reflected, especially when axolotls emerge from water post-metamorphosis. This challenge is met by the development of a tympanic middle ear, which transforms sound pressure in the air into particle motion in the inner ear fluid. In addition, the post-metamorphic middle ear contains an operculum. The opercularis muscle connecting scapulae and operculum was easily recognized in iodine-stained specimens of tiger salamanders and adult axolotls, but could not be found in the juvenile axolotls (17). Additionally, the urodele middle ear also contains the operculum, which is connected to the scapula of the shoulder girdle through the opercularis muscle and has been proposed to aid the transmission of substrate vibrations into the inner ear via the forelegs, plays a role in airborne hearing by bone conduction, or functions as a protective mechanism against loud sound exposures (18).

Bullock (19) first introduced non-invasive ABR recordings in fish. The ABR technique has since become widely used in auditory research. It has been applied to several vertebrate species, including fish and amphibians. ABR testing is particularly suitable for developmental studies in fish and salamanders due to its non-invasive nature and the lack of need for animal training (20).

Comparative studies of amphibian auditory systems can provide valuable insights into how terrestrial hearing evolved and how neural models have adapted to the selective pressures associated with communication. A key model for understanding vertebrate hearing (21) is the amphibian auditory system. The regeneration of hair cells that has been observed in amphibians, such as the axolotl, holds great promise for the development of therapeutic strategies aimed at restoring hearing health in humans.

METHODS

This study is an experimental research of auditory function in axolotls, before and after metamorphosis, and compares it with rats using electrophysiological measurements through ABR testing. A total of 10 3-month-old rats were obtained from the Medical Research Centre of the University of Medipol. For auditory evaluations, rats were anesthetized with ketamine (40 mg/kg, i.p.) and xylazine (Rompun, 10 mg/kg, i.p.). If needed, additional doses of ketamine (20 mg/kg, i.p.) and xylazine (5 mg/kg, i.p.) were administered to maintain anesthesia.

In the ABR test for rats, during the recordings, the active electrode was placed subcutaneously at the midline of the skull, the reference electrode beneath the pinna on the side where the hearing threshold was being determined, and the ground electrode under the contralateral pinna. The needle electrodes used (Ambu, Malaysia) were 0.40 mm in diameter and 12 mm in length. Testing began when electrode impedances were between 0 and 3 kΩ. Stimulus presentation and recordings were performed using an intelligent hearing systems (Miami, FL, USA) device, which was calibrated according to American National Standards Institute standards prior to the experiments. The stimuli used were 4 ms (rise time: 2 ms, plateau: 0 ms, fall time: 2 ms) tone bursts. The tone burst stimuli were presented at 8, 12, 16, and 20 kHz, using a Blackman envelope. The stimuli were delivered via high-frequency earphones designed for animal use. In all tests, a neonatal probe was used. The stimulus presentation rate was set at 19.3 stimuli per second, and 750 waveforms were averaged after amplification. A band-pass filter between 100 and 3,000 Hz was applied to the recordings, and the recording window was set to a total of 14 ms. At the end of the experiments, euthanasia was performed using a high-dose anesthesia protocol.

The axolotl specimens were obtained from the Medical Research Centre of the University of Medipol. In the present study, three axolotls were used for the recordings pre- and post-metamorphosis. We observed morphological and histological changes that are consistent with thyroid hormone-induced metamorphosis. The axolotls were given thyroid hormones and their metamorphoses were induced regardless of limb regeneration. The first Stage 1 animal was observed at day 8, and individuals completed metamorphosis (Stage 4) between day 28 and day 32 (22). The animals were housed in 5-liter containers. Their maximum length was recorded at 20 cm. Electrophysiological assessments were performed using ABR testing. This technique is widely used in humans and other animal models. Prior to testing, the axolotls were anaesthetized with a 0.025% benzocaine solution. Once anaesthetized, they were transferred to a test chamber. The test chamber was designed to minimize environmental and electromagnetic noise.

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During the test, the axolotls were placed on a hollow platform, and a wet towel was placed above and below them to maintain their viability before metamorphosis and facilitate skin respiration. The towels were re-moistened with fresh water every two minutes during the experiment to maintain optimal conditions for skin respiration, ensuring that the axolotls remained viable throughout the procedure. Electrophysiological responses were recorded using three stainless steel subdermal needle electrodes placed on the vertex (-), the ipsilateral mastoid (+), and the forehead (ground) (Figure 1). The ground electrode was placed on the animal's tail, the reference electrode on the nose tip, and the recording electrode on the forehead. The impedance values were measured between 0 and 1 kΩ but never reached 0 kΩ.

Responses were recorded using the Intelligent Hearing System with a gain of 100,000, a band-pass filter set between 100 and 3000 Hz, and a sweep count of 500. The rate was 11.1 Hz with an analysis time of 12 ms. Two trials were performed for each intensity level. An average response was calculated based on 1000 sweeps. Auditory stimuli consisted of tone bursts delivered through a bone vibrator at frequencies of 250 Hz, 320 Hz, 500 Hz, 600 Hz, 800 Hz, and 1000 Hz. In the earlier studies, the hearing was generally evaluated at low frequencies (17,23). The stimuli were monaural tone bursts with alternating onset phases and 4-8-4 ms rise-plateau-fall times. The Blackman envelope was used. The frequencies tested in axolotls and rats were selected based on their known auditory sensitivities and biological differences in hearing mechanisms. Axolotls are primarily sensitive to low-frequency sounds, as their hearing relies on bone conduction and particle motion detection rather than airborne sound waves. In contrast, rats have a well-established auditory range that extends into the high-frequency spectrum, typically between 1 kHz and 40 kHz, with most auditory studies focusing on mid-to-high frequencies (8 kHz-20 kHz), due to their natural sensitivity in these ranges. Therefore, the chosen frequencies for each species align with their natural auditory capabilities, ensuring a more accurate evaluation of their hearing function. The level of the stimulus was reduced step by step until the threshold was found. The axolotls were humanely euthanized with a highly concentrated benzocaine solution at the end of the experiments.

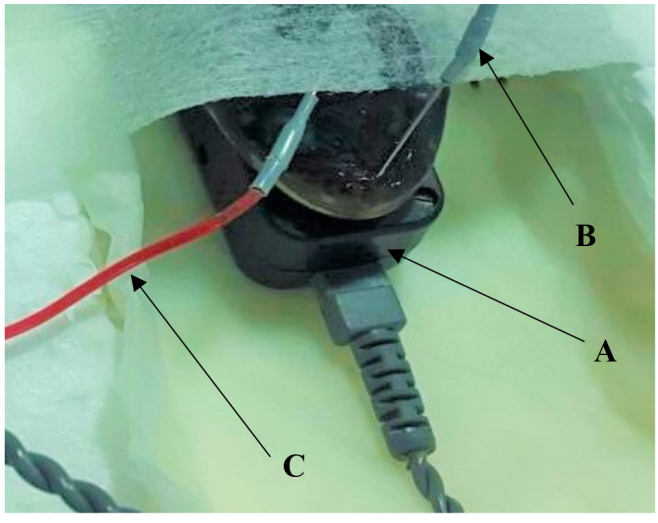

Figure 1. The experiment setup
A: Bone vibrator, B: Positive electrode, C: Negative electrode

For the first time, a bone vibrator (Radio Ear B71) was used as a transducer for an electrophysiological test system in axolotls in this study. The bone vibrator was carefully calibrated and positioned on the jaw bone of the axolotls to ensure that the same pressure was applied during all the recordings. To obtain reliable ABRs, it was essential to maintain constant pressure on the bone vibrator. To ensure this, a belt-like system was created and in each test the belt-like system was tightened the same amount. As the axolotls were all about the same size, the pressure applied to the test objects was consistent. The stimulus intensity was decreased in 5 dB SPL steps until the lowest detectable sound pressure level was reached. At this point, the auditory waveform became undetectable.

The difference in the methods of conduction used for the rats and the axolotls was because axolotls do not have conventional ears where classic transducers can be used for testing. To combat this, we had to use bone conduction. Because we wanted to compare the results of rats and axolotls, we aimed to standardize the results. We used air conduction for the rats as it is the accepted way of testing for these animals. The reason for this comparison is that we are familiar with the results of rats. We wanted to get an idea of the results of axolotls similarly by comparing the threshold morphologies.

Statistical Analysis

Due to the limited number of axolotl specimens used in this study, direct statistical comparisons between pre- and post-metamorphosis conditions could not be performed. Additionally, the different ABR testing methodologies used for rats prevented a direct comparison between rats and axolotls. As a result, only descriptive statistical analyses were conducted, including the calculation of mean and standard deviation for ABR thresholds and latencies. No formal inferential statistical tests, such as t-tests or one-way ANOVA, were employed in this study.

Informed Consent

This study did not involve human participants; therefore, informed consent was not required.

Ethical Statements

Approval for this study was obtained from the İstanbul Medipol University Animal Experiments Local Ethics Committee (decision no: 17, date: 10.02.2025).

RESULTS

All data presented in this study were collected using the Smart EPdevice from Intelligent Hearing Systems. In the auditory evaluations conducted on rats, ABR testing yielded distinct and reliable waveforms across all tested frequencies, thus demonstrating the efficacy of this methodology. The auditory

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thresholds for each frequency were determined, and clear ABR waveforms were recorded in response to tone bursts at 8, 12, 16, and 20 kHz. The lowest sound pressure level (dB SPL) at which a recognizable ABR waveform could be identified was defined as the threshold. Figure 2 illustrates a representative ABR waveform for one of the frequencies, while Table 1 presents the threshold values for each frequency. Additionally, Figure 3 illustrates the comparison of ABR thresholds between the right and left ears across the tested frequency spectrum. These results provide a baseline understanding of auditory function in rats, facilitating a comparative analysis with other species. The observed waveforms were consistent and demonstrated typical auditory processing patterns across the frequency spectrum.

The use of a bone vibrator for evoked ABR testing in axolotls is the first of its kind in the literature. As a result, we have been able to test this novel technique in this rare species. Recognizable responses were obtained for all of the stimulus frequencies that were tested. Among the bursts used, the 600 Hz burst elicited the best waveform morphology (Figure 4).

The thresholds for the 600 Hz tone bursts pre- and post-metamorphosis were 55 dB SPL and 58 dB SPL, respectively. These thresholds are indicated by an asterisk (Figures 3,4). To ensure consistency of the waveforms, multiple traces were recorded. The mean hearing threshold for the axolotl was found to be 55 dB SPL. One of the most critical findings was the longer ABR latencies recorded pre-metamorphosis, averaging 1.5 ms, compared to post-metamorphosis (Figure 4).

Figure 5 shows the effect of decreasing stimulus intensity (from 70 to 40 dB SPL). As intensity decreases, wave amplitudes decrease and wave latencies increase. In this recording, the threshold of hearing at 250 Hz was found to be 50 dB SPL. The neurogenic characteristics of the auditory responses are reflected in these results. Another significant finding is that the prolongation of wave latencies, compared to humans and rats, is less pronounced in axolotls. Figures 6 and 7 show the objective ABR thresholds.

Figure 2. Comparison of ABR thresholds between the right and left ears in rats across different frequencies (8, 12, 16, and 20 kHz)
ABR: Auditory brainstem response

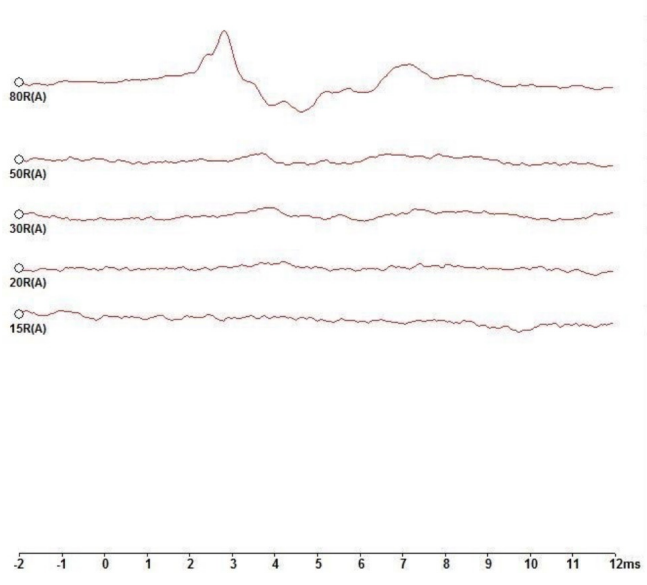

Figure 3. Representative ABR waveform in a rat at 8 kHz. The waveform shows the distinct peaks used to determine the hearing threshold. The threshold is marked with an asterisk
ABR: Auditory brainstem response

Table 1. ABR thresholds (dB SPL) for each frequency tested in rats. The table summarizes the minimum sound pressure levels at which ABR waveforms were identifiable

	Mean	Standard deviation	Minimum	Maximum
8 kHz Left	21.0	±5.67	10	25
12 kHz Left	17.0	±4.21	10	25
16 kHz Left	21.5	±3.37	15	25
20 kHz Left	5.5	±6.43	0	20
8 kHz Right	20.5	±3,68	15	25
12 kHz Right	15.5	±2.83	10	20
16 kHz Right	22.0	±2.58	20	25
20 kHz Right	5.5	±4.97	0	15

ABR: Auditory brainstem response

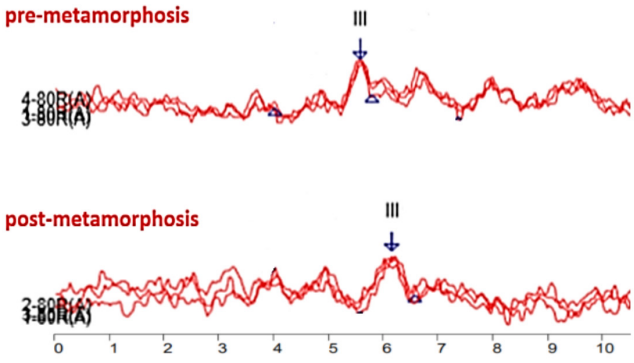

Figure 4. ABR result made at a frequency of 600 Hz. Recordings were performed for 3 times to prove the reliability of the ABR waveforms
ABR: Auditory brainstem response

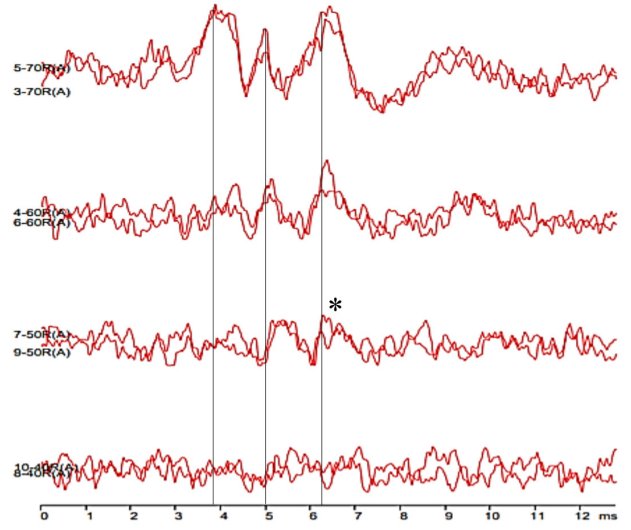

Figure 5. ABR waveforms recorded at a frequency of 250 Hz of Axolotl post-metamorphosis. The threshold is marked with an asterisk
ABR: Auditory brainstem response

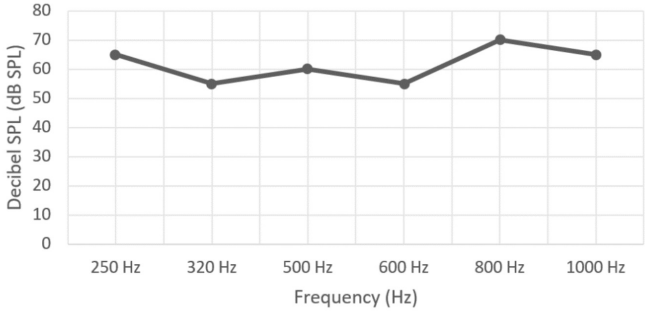

Figure 6. Estimated objective thresholds of an Axolotl pre-metamorphosis

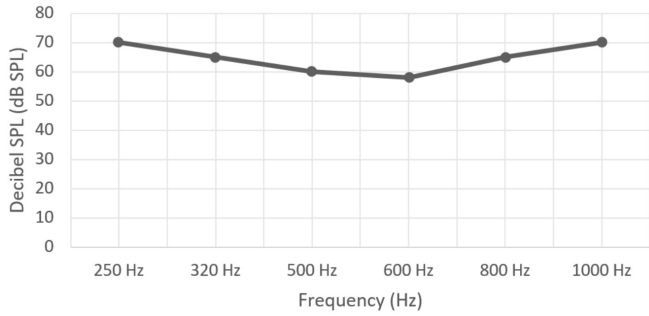

Figure 7. Estimated objective thresholds of an Axolotl post-metamorphosis

DISCUSSION

The primary aim of this study was to investigate the effects of metamorphosis on the auditory system in axolotls. Auditory evoked potentials (AEPs) were used to assess the animals' ability to perceive vibrating sounds. Metamorphosis induces several changes in the axolotl body. One of the systems that undergoes changes is the auditory system. For example, the columella auris is free pre-metamorphosis. Post-metamorphosis, it fuses with the otic capsule (17).

Pre-metamorphosis, the latencies of AEPs were longer than those recorded post-metamorphosis. While the precise mechanisms underlying this observation remain unclear, it is likely that both anatomical and physiological changes during metamorphosis contribute to this shift. One hypothesis is that the fusion of the columella auris with the otic capsule post-metamorphosis may alter the conduction pathway for sound waves, leading to changes in latency and threshold values. Additionally, the development of the tympanic middle ear could introduce structural changes that modify how sound pressure is transformed into particle motion within the inner ear fluid.

At the cellular level, metamorphosis could impact the density and arrangement of hair cells within the inner ear, potentially influencing how auditory signals are transmitted to the brainstem. Research on other amphibians and vertebrates has shown that changes in the mechanical properties of auditory structures, such as the ossicles or tympanic membrane, can affect auditory sensitivity and latency. Further histological and molecular studies on the inner ear structures pre- and post-metamorphosis would be necessary to identify the specific alterations driving these functional changes in axolotls.

Furthermore, the amplitudes were higher post-metamorphosis. However, the amplitude might be partly dependent on the pressure exerted on the animal by the bone vibrator. Metamorphosis is a process of many changes in AER characteristics would be expected. Factors such as cranial bone density, tissue density and skin permeability may all have an effect.

Following metamorphosis, sound has more difficulty reaching the inner ear because of the increased impedance mismatch between air and body skin. The delay in auditory evoked responses

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observed post-metamorphosis (16) may be partly explained by this. However, more detailed research is needed in this field.

This study has successfully demonstrated the feasibility of conducting objective auditory testing in axolotls using ABR testing for the first time in this species, employing a bone vibrator as a transducer. The findings show that metamorphosis induces significant changes in the axolotl auditory system, as evidenced by increased ABR thresholds and altered latencies. Specifically, the longer latencies observed pre-metamorphosis suggest fundamental physiological changes that affect auditory processing post-metamorphosis. These results indicate that metamorphosis leads to increased impedance and modifications to the middle ear structure, contributing to changes in sound transmission.

The successful use of a bone vibrator to measure auditory responses in axolotls is a noteworthy outcome of this study, providing a reliable method for assessing auditory function in amphibians. Additionally, the identification of 600 Hz as the most effective frequency for ABR recordings both pre- and post-metamorphosis further solidifies this technique's potential utility in future research on auditory systems in regenerating species.

The findings from this study offer promising insights not only for amphibian auditory research but also for broader applications in regenerative medicine and auditory system repair. Axolotls' ability to regenerate inner ear structures pre-metamorphosis provides a unique opportunity to explore the molecular and cellular processes underlying hair cell regeneration, which could inform future strategies for hearing restoration in humans. In mammals, the loss of regenerative capacity in hair cells leads to irreversible hearing loss. Understanding the factors that enable axolotls to regenerate these cells and identifying what changes occur during metamorphosis that halt this process could yield critical knowledge applicable to therapeutic interventions. Furthermore, the use of a bone vibrator for ABR testing establishes a non-invasive method that could be applied to other regenerative models or even species that undergo auditory changes as part of their development.

In addition to the findings from axolotls, the auditory tests conducted on rats yielded consistent ABR waveforms across the tested frequencies (8, 12, 16, and 20 kHz). The determined thresholds reflect stable auditory processing in rats, offering a useful point of comparison with the auditory responses of axolotls. While axolotls exhibited alterations in auditory function subsequent to metamorphosis, the rats exhibited consistent auditory thresholds across frequencies, thereby establishing them as a reliable model for baseline auditory function. This contrast serves to underscore the potential for comparative studies to elucidate species-specific auditory mechanisms.

Future studies could investigate how regenerative capacity varies across different developmental stages and how auditory function is restored post-injury, potentially leading to breakthroughs in treating auditory damage caused by trauma or age-related degeneration.

Study Limitations

This study has several limitations. First, the small sample size of three axolotls may restrict the generalizability of the findings. Additionally, the absence of molecular or histological analyses limits the understanding of the cellular mechanisms underlying the changes in auditory function observed pre- and post-metamorphosis. Lastly, variability in bone vibrator pressure could have influenced the amplitude of ABR measurements, suggesting a need for further investigation to ensure consistent pressure application across developmental stages and different specimens.

CONCLUSION

This study is the first to apply ABR testing with a bone vibrator in axolotls, evaluating auditory function before and after metamorphosis. Although different frequencies and conduction methods were used for axolotls and rats due to species-specific differences, a comparison was necessary to interpret and set a base point for the axolotl data. Aware of this limitation, we selected rats and air-conduction ABR-one of the most widely accepted methods in hearing research-as a scientific reference point. This approach enabled a meaningful comparison and revealed significant changes in axolotl auditory thresholds and latencies following metamorphosis. These findings support the feasibility of objective auditory testing in axolotls and highlight their potential as a model in auditory and regenerative research.

Ethics

Ethics Committee Approval: This study was approved by the Istanbul Medipol University Animal Experiments Local Ethics Committee (decision no: 17, date: 10.02.2025).

Informed Consent: This study did not involve human participants; therefore, informed consent was not required.

Footnotes

Author Contributions: Surgical and Medical Practices - F.B.; Concept - F.B.; Design - F.B., M.B.Ş; Data Collection and/or Processing – F.B.; Analysis and/or Interpretation- F.B., Ş.T.Ö., M.B.Ş; Literature Search - F.B., Ş.T.Ö; Writing - F.B., Ş.T.Ö.

Conflict of Interest: The authors have no conflict of interest to declare.

Financial Disclosure: The authors declared that this study has received no financial support.

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