

Hearing Capabilities of the Atlantic Mudskipper (*Periophthalmus barbarus*) Across Multiple Media.

By

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ABSTRACT

An animal's sensory detection mechanisms are often shaped by the environment in which that animal lives. Amphibious fishes provide a rare opportunity to study how hearing has adapted to match the extremely unique lifestyle of a fish that spends much of its time out of the water. This study compared the hearing capabilities of the amphibious Atlantic mudskipper (*Periophthalmus barbarus*) with that of the fully aquatic banded sculpin (*Cottus carolinae*). Auditory brainstem response testing was performed on individuals of the two species in three different settings: air, substrate, and substrate underwater. Our study found that no *P. barbarus* nor *C. carolinae* individuals were able to detect airborne auditory stimuli. *P. barbarus* and *C. cottus* individuals were unable to detect substrate-borne vibration at frequencies of 800Hz and 1600Hz. Evoked potentials for *P. barbarus* and *C. carolinae* were nearly identical. *P. barbarus* is unable to hear airborne stimuli and has a similar audiogram to a fully aquatic fish, *C. carolinae*, which suggests that its auditory capabilities have not experienced adaptations as a result of their amphibious lifestyle.

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List of Abbreviations

ABR - auditory brainstem response

AEP – auditory evoked potential

INTRODUCTION

Communication plays an important role in many animal taxa. Acoustic communication is vital within animal communities as it is necessary for sexual reproduction and agonistic interactions (Bradbury and Vehrencamp 1998, Kierl and Johnston 2010). Therefore, how well an animal communicates has a direct impact on the fitness of that animal. Acoustic communication has been studied in many animal taxa including birds (Slater 2003), mammals (Boughman 1997, McComb et al. 2003), and amphibians (Gerhardt 2001). In aquatic environments, the acoustic communication of marine mammals (Edds-Walton 1997, Janik and Sayigh 2013) has received much of the academic attention. Despite fish making up most vertebrate biodiversity (Nelson 2006), comparatively little research has been done on this group relative to marine mammals.

Fishes have developed more methods of sound production than other vertebrate group (Ladich and Fine 2006). This is due, in part, to the fact that acoustic communication is harder to interrupt in water than other communication strategies such as visual or chemical communication, and also due to their diversity. Lotic aquatic environments can fluctuate between being clear and turbid depending on many factors, so the success of visual communication can be interrupted by the environment. Similarly, lotic environments have continually flowing water which can prevent chemical communications from reaching their target effectively (Bradbury and Vahrenkamp 1998, Cruz and Lombarte 2004). Acoustic communication by fishes has been recognized since Aristotle (Parker 1918). Research on sound-producing mechanisms started in the 1800's with Müller and Dufossé. It was during this period that pectoral fin stridulation in catfish (Müller 1857) and contractions of muscles around the swim bladder were discovered to be sources of sound production (Dufossé 1874). Currently, the most common forms of sound production in fishes are through compressions of the swim bladder using sonic muscles and

pectoral fin adaptations. Other sound producing mechanisms in fish include vibrating the pectoral girdle in some cottids (Barber and Mowbray 1956), grinding of pharyngeal teeth in many families (Bertucci et al. 2014), plucking of tendons against bony structures (Kratovichil 1978), and the expulsion of bubbles through the anus seen in Clupeids (Wahlberg and Westerberg 2003).

Sound in water takes the form of a wave of pressure that is moving in all directions away from the source of the sound. As the wave of pressure moves throughout the water, the water particles are displaced and bounce back and forth, thus creating particle motion. Therefore, sound can be detected in water via pressure wave and/or particle motion (Popper et al. 2019).

Fishes detect sound by using two structures, those being the inner ear and the neuromast system. The inner ear within fishes consists of three semicircular canals and three otolith end organs. Within these two sets of organs, the otolith structures are associated most strongly with hearing (Ladich and Popper 2001). The inner ear is composed of three canals that lead to calcified structures called otoliths (Popper et al. 2019) The otoliths reside within the otolithic membrane and are connected to the membrane wall with hair-like structures that contain sensory cells. The otolith is a hard, calcified structure and acts similarly to an accelerometer. A fish's body is close to the same density as water, so a fish's otoliths are going to be denser than the rest of the fish's body. This means that as vibration passes through a fish, the otolith is going to vibrate at a different magnitude than the rest of the fish's body. The hair-like sensory cells that connect the otolith to the surrounding membrane will trigger at this difference in vibration, which will be interpreted by the fish's brain as sound (Popper and Fay 2009). The otolith in most fish would primarily be used to detect particle motion. (Popper et al. 2019). This means that the otolith would be more effective in the near-field rather than the far-field, because the near-

field is where particle motion is most prominent (Ladich and Popper 2008). For a fish to be effective at interpreting far-field noise, they would need to be sensitive to pressure waves. This points to fish with swim bladders that have an adaptation to boost hearing sensitivity by connecting the swim bladder to the inner ear as being most suited for far-field hearing (Ladich and Popper 2008). Otolith size can provide further insight into a fish's acoustic capabilities. Cruz and Lombarte (2004) found that Sciaenids (fish with large otoliths) frequently produce sound, which indicates that they specialize in acoustic communication; whereas Labrids (fish with relatively small otoliths) are frequently brightly colored which indicates a specialization in visual communication (Cruz and Lombarte 2004). While hearing is highly variable in fishes, the inner ear is typically able to hear at frequencies up to 800Hz-1000Hz (Popper et al. 2019).

The second tool used by fish to detect sound is the neuromast system. A neuromast is a bundle of sensory hairs that are held within a gelatinous dome called a cupula. A neuromast is able to detect sound when the sensory hairs are triggered by the movement of the cupula as a result of particle motion. The neuromast system can be categorized by the two types of neuromasts that are found on a fish, those being lateral line neuromasts and superficial neuromasts. Lateral line neuromasts, also called canal neuromasts, reside within the fish's lateral line. Superficial neuromasts are found scattered elsewhere on the body and are positioned on the surface of the fish (Mogdans 2019). These neuromasts operate much like those in the inner ear in detecting sound. The neuromasts primarily contribute to hearing sensitivity at lower frequencies, ~100-200Hz. Higgs and Radford (2013) found that goldfish who have had their lateral line disabled had a 10-15dB increase in their hearing thresholds at 100Hz and 200Hz but did not have a difference at higher frequencies (Higgs and Radford 2013).

While all fish are capable of receiving auditory stimuli via particle motion, some species of fish that have a swim bladder, typically used for buoyancy control, have a set of bones called “Weberian ossicles” that connect the swim bladder to the inner ear. These fish belong to the Superorder Ostariophysi and have been referred to as “hearing specialists” (Popper and Fay 2011). By having a swim bladder that is connected to the inner ear, ostariophysian fishes are able to receive sound via pressure waves. This is due to the swim bladder being an air-filled sac that is of a lower density than the surrounding environment, meaning that it compresses and expands when pressure waves pass through it (Popper and Fay 2011). Other fishes have a swim bladder that is connected to the inner ear without the presence of Weberian ossicles. Some have a swim bladder that comes into direct contact with the posterior portion of the inner ear; others, while other fish have gas filled tubes that run directly under the inner ear from the swim bladder (Allen et al. 1976, Coombs and Popper 1979). This added strategy of hearing allows hearing specialists to hear a wider range of frequencies at lower intensities (Amoser and Ladich 2005). After creating the distinction of hearing specialist and hearing generalists, the authors of that paper, Arthur Popper and Richard Fay, have since suggested that the terms be dropped (Popper and Fay 2011). The authors recommend describing the specific specialization the fish in reference possess instead of simply labelling the fish a “hearing specialist.”

Although hearing and sound production have been studied in a number of fishes, few studies have looked at acoustic adaptations to unique lifestyles/habitats. An exception is a hearing study that investigated if cavefish have any hearing adaptations as a result of their subterranean environment. This study compared two obligate cave species, *Typhlichthys subterraneus* and *Amblyopsis splaea*, and one surface-dwelling species, *Forbesichthys agassizii*. This study found that both *T. subterraneus* and *A. splaea* had a reduced hearing range. These

obligate cave-dwelling species had similar hearing capabilities at lower frequencies (100Hz-800Hz) but had lost the ability to hear at frequencies about 800Hz, whereas the surface-dwelling species can hear frequencies up to 2000Hz. The loss of hearing at high frequencies was a result of loud background noise that was present at frequencies greater than 800Hz. The background noise was caused by the ripples that existed in the cave and water droplets falling off the cave ceiling. The high-frequency background noise would likely mask attempts at communicating at high frequencies, so the cavefish species no longer needed to hear at that frequency (Niemi et al. 2013).

The Atlantic mudskipper (*Periophthalmus barbarus*) was first described by Linnaeus in 1766. This species is native to the Atlantic coast of Africa, ranging from Senegal to Angola (Fischer et al. 1981) and is the sole representative of the *Periophthalmus* genus in west Africa (Etim et al. 2002). Mudskippers are proficient burrowers, constructing tunnels in mudbanks that they use as refuge from harsh temperatures, safety from predators, and for brooding eggs (Ishimatsu and Gonzales 2011). Mudskippers are preyed upon in both environments. Their predators include birds, snakes, and mammals while on land and by larger fish while in the water (Swanson and Gibb 2004). Mudskippers primarily consume invertebrates such as polychaetes, crabs and insects during low tide (Turay et al. 2006).

What makes the Atlantic mudskipper so fascinating from a behavioral standpoint is that it belongs to one of the few truly amphibious groups of fish. This means that it possesses adaptations that allow for a partially terrestrial existence (Murdy 1989). Research into mudskipper biology has shown several adaptations to assist with their time spent out of the water. Mudskippers possess adaptations to their eyes which allow for accurate sight in air as a result of a strongly curved cornea and flattened lens. By having a curved cornea and a flattened

lens, mudskippers are able to better see through the air, resulting in normal or even far-sighted vision. (Stebbins and Kalk 1961, Sponder and Lauder 1981). It is suggested that mudskippers primarily detect prey through vision. Observations reported that during terrestrial hunting, mudskippers would halt and stand still for a short period after seeing prey before lunging on their prey (Stebbins and Kalk 1961). Mudskippers also use their improved vision through the air to detect predators (Stebbins and Kalk 1961). While residing in shallow water, mudskippers will keep their eyes above the surface of the water to detect predators (Tytler and Vaughn 1983). Mudskippers are able to use their pectoral fins for both aquatic and terrestrial locomotion (Pace and Gibbs 2009). Mudskippers exhibit a locomotive strategy called “crutching.” This is performed by using the pectoral fins to lift the mudskipper’s center of mass of the substrate and propel it forward (Pace and Gibb 2009). Terrestrial movement is made possible by modified pectoral fins which have two joints, a joint that connects the cleithrum to the pectoral radials, analogous to a shoulder joint, and a second joint within the pectoral fin between the radials and rays (Pace and Gibb 2009). Mudskippers exhibit separate strategies for eating prey in aquatic and terrestrial environments. Mudskippers are able to use suction to capture prey in aquatic environments. In terrestrial environments, mudskippers use water held in their buccopharyngeal cavity, released over the prey, and then withdrawn back into the mudskipper, thus capturing the prey in their mouth (Michel et al. 2016). Mudskippers exhibit an adaptation that allows for terrestrial respiration in the form of a highly vascularized buccopharyngeal cavity (Aguilar et al. 2000).

Mudskippers are known to produce sounds during agonistic encounters and for courtship (Polgar et al. 2011). *Periopthalmodon septemradiatus* have been recorded using a combination of pulses and tones in combination during experiments simulating territorial bouts. The main

frequency of pulses produced by *Pn. septemradiatus* was 46Hz. The main frequency of tones produced by *Pn. septemradiatus* was 162Hz (Polgar et al. 2011). The sounds produced by the mudskippers were often paired with behavior displays. These displays included presenting dorsal fins, opening mouth wide (gaping), and darkening body coloration. Display of dorsal fins, darkening body coloration, and gaping were the most common behaviors during agonistic display (Polgar et al. 2011). While actions such as tail standing/jumping, tail twirling, and gaping were commonly performed by males in courtship displays (Clayton and Townsend 2007).

Hearing adaptations in mudskippers are understudied relative to other physiological adaptations. A species' hearing capability is primarily shaped by the environment in which that species exists (Endler 1992). This raises the question of how an amphibious fish has adapted to receive sound. Animals that communicate through the air must overcome the impedance mismatch of the low impedance of air and the high impedance of the inner ear, while animals that receive sound through water or substrate do not have to worry about this. Impedance matching is usually resolved by the presence of an anatomical structure that connects a tympanic membrane directly to the inner ear, like a columella in amphibians and reptiles, or the middle ear in humans (McGill 1960). Since fish lack this anatomical structure, how would a fish that spends a large portion of its day out of the water receive sound?

One potential mode of hearing that would fit the amphibious lifestyle of the mudskipper would be substrate-borne hearing. This would make sense from both an anatomical perspective and a lifestyle perspective. Mudskippers have no swim bladder, so most of their time in water would be spent benthically. When on land, mudskippers are subjected to gravity; so, in both aquatic and terrestrial environments, mudskippers would spend almost all of their time connected to the substrate. The idea of mudskippers favoring substrate-borne hearing is strengthened by

several factors. Mudskippers are able to communicate through the substrate while out of the water. Polgar (2011) reported that *Pn. septemradiatus* communicated using a combination of low frequency (under 200Hz) tones and pulses while out of the water, using damp, substrate as the media (Polgar et al. 2011). Using substrate as the medium to produce calls would imply that those sounds would be received via the same medium. Mudskippers have an increased density of superficial neuromasts along the ventral side of their head (Harms 1929, Polgar 2011). This would allow for increased sensitivity to low frequency particle displacement on the surface of the substrate (Polgar et al. 2011, Higgs and Radford 2013). Substrate-borne hearing would be the one media through which a mudskipper would almost always be in contact with. Due to their amphibious nature, utilizing either air or water as the primary media through which to hear would leave the mudskipper deaf either while they are on land feeding and courting mates, or in their burrows mating and brooding eggs. It is hypothesized that mudskippers primarily use vision to detect prey (Stebbins and Kalk 1961). It is hypothesized that mudskippers also primarily use their vision when detecting predators (Stebbins and Kalk 1961, Tytler and Vaugh 1983). So intraspecific interactions like territory defense and courtship appear to be the main role that acoustic production and hearing plays in the daily life of a mudskipper.

The objective of this study was to examine hearing capabilities of *Periophthalmus barbarus* to investigate the possibility of a unique hearing adaptation to terrestrial life by testing it's sensitivity to aerial auditory stimuli, and comparing auditory evoked potential across multiple media to the auditory evoked potentials of a fully aquatic fish, *Cottus carolinae*.

RESEARCH GOALS

To determine if *P. barbarus* have unique hearing capabilities as a result of their amphibious lifestyle, this project sought to answer two questions:

1. Can *Periophthalmus barbarus* detect airborne stimulus?
2. Will *Periophthalmus barbarus* have different hearing capabilities than a fully aquatic fish, *Cottus carolinae*?

We hypothesize that:

1. *P. barbarus* will not detect airborne stimulus.
2. *P. barbarus* will have different hearing capabilities to a fully aquatic fish.

METHODS

Animal collection and husbandry

The reason we chose *C. carolinae* to use as a comparison group for *P. barbarus* is because there has been prior research done that suggests a similarity in acoustic communication between sculpin and gobies (Zeyl et al. 2016). Also, neither *P. barbarus* nor *C. carolinae* have a swim bladder, this is ideal because it allows us to compare the hearing capabilities of these fish without worrying about organs that amplify hearing capabilities. By choosing *C. carolinae* as our outgroup specimen, we were able to ensure that if there are any differences in hearing, it is a result based on the amphibious lifestyle of *P. barbarus* and not an extraneous factor.

Periophthalmus barbarus individuals were purchased through the aquarium supply company liveaquaria.com. *P. barbarus* individuals ranged in size from 68mm total fish length to 110mm total fish length. *Cottus carolinae* individuals were collected from coldwater creek; a part of the Coosa river drainage, in Anniston, AL (33.597762, -85.923640). *C. carolinae* individuals ranged in size from 72mm total fish length to 117mm total fish length. *P. barbarus*

individuals were kept in aquaria at a density of 2 individuals per 75.7-liter aquaria. Each aquarium was set up such that half the tank was aquatic, and half the tank was terrestrial, with the substrate distributed in a slope so that *P. barbarus* individuals can easily move between aquatic and terrestrial environments. Water temperature was kept at 28°C. The pH of the water was kept between 7.0 and 8.5. Salinity was kept at ~1.007 sg. Aquarium substrate was composed of sand. Sand was piled on one side of the tank to form a beach. One 10cm-by-10cm ceramic aquarium tile per mudskipper was placed in each aquarium and propped against the side of the aquarium to create a shelter. *P. barbarus* individuals were fed blood worms every other day. Each aquarium was given 28.3 grams of blood worms. After 1 hour, any blood worms that remained in the tank were removed to prevent detrimental impacts on water quality. *Cottus carolinae* individuals were kept in 75.7-liter aquaria with gravel beds at a density of three individuals per aquarium. Each aquarium had one 10cm-by-10cm ceramic aquarium tile per sculpin to provide a shelter for each individual. The water had highly oxygenated, frequently filtered water. Water temperature was kept at 20°C with a pH between 6.5-7.5. *C. carolinae* were fed one live earth worm every other day. We attempted to feed the sculpin blood worms but they only responded well to live food. *P. barbarus* and *C. carolinae* individuals were kept under a 12-hour diel cycle. No research has been done into the role of size of hearing sensitivity for the two fish in this study, but there has been some research done on this topic with zebrafish, *Dania rerio*. This study suggested that there is no significant impact of size on hearing sensitivity (Higgs et al. 2001).

Audiometry

All sound tests were conducted in a glass bowl that had a 177.8-mm diameter and a depth of 190.5-mm (Figure 1). Within the bowl, fish were secured to a block of clay that was 114.3-

mm tall, 76.2-mm wide, and 38.1-mm tall. Fish were secured to the clay block using straight pins positioned around the fish's body and wires bent into a U-shape and positioned over the fish to restrain the fish from moving laterally and vertically. Airborne stimuli were sent through an amplifier (Behringer Europower EPQ304) and presented to the animals via a 203.2-mm diameter speaker (MTX midbass driver) (Figure 1). The speaker was positioned 342.9-mm behind the head of the fish being tested. Ground-borne stimuli were sent through the same amplifier and presented to the animals through a bass shaker (Aurasound AST-2B-4 Pro) (Figure 1) that was glued to the bottom of the glass testing bowl. For submerged ground-borne trials, 152.4-mm of water was present in the bowl. For airborne and dry ground-borne trials, 12.7-mm of water was put in the bottom of the glass bowl to function as a reservoir for a respirator. The testing apparatus was positioned on top of several layers of foam to minimize the presence of external vibrations. A plastic pipette (Corning costar 10mL in 1/10 graduated pipette) (Figure 1) that was bent to form a right angle positioned in the fish's mouth in order to prevent the fish from moving forward out of the restraints. During airborne and out-of-water ground-borne trials, a respirator was used to keep water moving over the fish's gills. Plastic tubing was placed in the bottom of the glass testing bowl which led to a 12V peristaltic pump (Gikfun AE1207). The pump drew water from the reserve that was kept in the bottom of the glass testing jar. The peristaltic pump was connected via plastic tubing to the plastic pipette that acted as a mouthpiece. The respirator apparatus was powered by a 12V battery (Universal Ub1280 sealed lead acid battery).

The signals that were used as auditory stimuli were generated in Audacity. Ground-borne tones were 100hz, 200hz, 400hz, 800hz, and 1600hz; with a decibel range of 45dB re 1 μ Pa to 115dB at 5dB intervals. Each decibel level was presented to the fish twice, once each at opposing phases (90° and 270°). Ground-borne tones were 0.30 seconds in duration and did not show any

gradual onset or offset of amplitude. Ground-borne stimuli had a 300ms interstimulus interval between each tone to allow for the vibrations of the previous tone to dissipate before the next tone started. Each phase of each decibel level of ground-borne stimuli lasted a total of 105 seconds. Airborne tones were 100hz, 200hz, 400hz, 800hz, and 2000hz; with a decibel range of 20dB re 1 μ Pa to 105dB at 5dB intervals. Airborne tones had a duration of 0.20 seconds and had a 0.005 second fade in/out. Airborne stimuli had a 46ms interstimulus interval between each tone. Each phase of each decibel level of airborne stimuli had a duration of 30 seconds. Signals were sent to the amplifier from Superlab 5.0.

A calibration file was created for each frequency. This calibration file was used as the reference point against which testing frequencies were adjusted. This was done to guarantee that each tone was correctly testing the frequency and volume that it was labeled as. Peak voltage (mV) of the calibration files were recorded via the digital oscilloscope (Tektronix TBS 1052B). Subsequent files were then adjusted using a volume control knob (Behringer MONITOR1) to match the peak voltage of the calibration files. For ground-borne stimulus, an accelerometer was screwed into the clay block within the glass testing jar via a metal ring implanted in the clay block. This accelerometer was connected to the digital oscilloscope in order to measure the voltage of the calibration files. For airborne trials, a microphone (Behringer B5) was connected to the digital oscilloscope via an audio recorder (Tascam DR-60D). The microphone was positioned where the fish's head would be in a trial and was used to record the voltage of the airborne calibration files in order to properly adjust them. Tones were calibrated at the beginning of each day.

Electrodes were used to record the brain activity of the specimens in response to the auditory stimuli. Electrodes were insulated using a layer of nail polish on all but the very tip of

the electrodes. Two electrodes were positioned in front of the testing station. One of these electrodes (reference electrode) was inserted under the specimen's skin by the fish's nares. A second electrode was positioned posterior to the skull (recording electrode) in close proximity to the brain. A third electrode (ground electrode) was placed in subdermally beside the second dorsal fin of the specimen. These electrodes were then connected to a data acquisition system (BIOPAC MP35) that was used to visualize and analyze the electric signals produced by the specimen's brain in response to auditory stimuli. Auditory brainstem response (ABR) data was visualized and analyzed using Biopac student 4.1 software. ABR data was passed through a 60Hz notch filter. In order to cancel potential stimulus artifacts, each stimulus was presented to the fish twice, at opposing phases (90° and 270°). Recordings from the two opposing phases of each decibel level were then combined into a single waveform by averaging them together. The auditory evoked potential (AEP) was defined as the lowest decibel level at which the fish gave a repeatable response. The waveform was visually assessed for peaks that could be clearly differentiated from background noise in order to determine the lowest sound level at which an AEP occurred. The "peak" in the waveform that is visually interpreted is an increase in electrical activity in the brain which represents an increase in brain activity as a result of auditory stimuli. Evoked potentials were recorded from a dead mudskipper so that a baseline background noise waveform could be used as a comparison for the test fish. Responses were confirmed to be true by analyzing the following sound levels for an evoked potential that occurs with less lag time and/or with progressively increasing evoked potential intensity.

Statistics

Species threshold were compared using one-way repeated measures ANOVAs. Species group was the between-subject factor and frequencies at 100, 200, and 400Hz were the repeated

measures factor. Total fish length was included as a covariate. Tukey's post-hoc test was used to examine the results of the ANOVAs. Total length did not prove to be significant, so it was removed from the model. All statistics were performed in SPSS statistical software.

RESULTS

Representative waveforms are included from aerial (Figure 2A), exposed substrate-borne (Figure 2B), and submerged substrate-borne (Figure 2C) trials from an individual *P. barbarus* at 100Hz visually represent the process of a fish reaching its hearing threshold. Evoked potentials increased in intensity as decibel level increased after the fish reached threshold.

Neither *P. barbarus* individuals, nor *C. carolinae* individuals reached an auditory threshold during airborne ABR trials at 100hz, 200hz, 400hz, 800hz, and 2000hz frequencies. Because none of the fish reached an air borne hearing threshold, we had no data to analyze. Waveforms from aerial trials of *P. barbarus* are included.

Within submerged substrate-born trials, 100Hz was the frequency at which both species groups had the most acute auditory response with an average threshold of 90.83dB (n = 6) for *P. barbarus* and an average threshold of 89dB (n=5) for *C. carolinae*. These thresholds were not significantly different (95% CL -2.518 – 6.185; P=0.365). The average threshold for 200Hz within submerged substrate born trials were 93.33dB (n=6) for *P. barbarus* and 94.0dB (n=5) for *C. carolinae*. These thresholds were not significantly different (95% CL -5.308 – 3.975; P=0.753). The average threshold for 400Hz within submerged substrate-born trials was 100Hz (n=1) for *P. barbarus* and 98.33dB (n=3) for *C. carolinae*. These thresholds were not significantly different (95% CL -36.28 – 39.61; P=0.868). It should be noted that for submerged substrate-born trials, only 1 *P. barbarus* individual and only 3 of the 5 *C. carolinae* individuals

had a response at 400hz. Neither *P. barbarus* nor *C. carolinae* reached a hearing threshold at 800Hz or 1600Hz during submerged substrate-borne trials.

Within dry substrate-borne trials, 100hz was once again the frequency at which both species groups had the most acute average auditory response in which *P. barbarus* had an average threshold of 90.0dB (n=6) and *C. carolinae* had an average threshold of 87dB (n=5). There was no significant difference between these thresholds (95% CL -2.206 – 8.606; P=0.225). The average threshold for 200Hz frequency with dry substrate-borne trials were 93.33dB for *P. barbarus* (n=6) and 90dB (n=5) for *C. carolinae*. There was no significant difference between these thresholds (95% CL -19.39 – 8.606; P=0.186). The average thresholds for 400Hz frequency within dry substrate-borne trials were 98.33dB (n=3) for *P. barbarus* and 107.5 (n=2) for *C. carolinae*. There was no significant difference between these thresholds (95% CL -18.23 – 0.108; P=0.49). It should be noted that within dry, substrate-borne trials, only 3 of the 6 *P. barbarus* individuals had produced a response, and only 2 of the 5 *C. carolinae* individuals had a response. Neither *P. barbarus* nor *C. carolinae* reached a hearing threshold at 800Hz or 1600Hz during exposed substrate-borne trials.

DISCUSSION

Our results support our hypothesis that, despite the amount of time that *P. barbarus* individuals spend outside of the water due to their amphibious lifestyle, this species does not possess the necessary adaptations to receive sound through the air. Due to *P. barbarus* lacking a form of impedance-matching structure, it would be difficult for the species to convert airborne pressure stimuli into mechanical displacements required by the inner ear. Airborne vibration exists in a low-density media of air, and to be interpreted by the inner ear that exists in higher

density fluid, it must travel through an impedance-matching structure such as a columella that exists in the amphibian ear (McGill 1960).

For an animal that spends so much of their time on land, the lack of sensitivity for airborne sound is somewhat surprising even if mudskippers appear to possess no impedance-matching structure. One possible reason for a lack of airborne hearing is that mudskippers are more effective at using their visual prowess to hunt food and detect predators, and the need for airborne hearing did not necessitate the energy and evolutionary investment to form the required impedance-matching structure (Tytler and Vaughn 1983, Wright and Turko 2016). Mudskippers are proficient at spotting and catching prey. Their eyes are situated on the top of their head so that they are granted good vision in all directions. Mudskippers can see a partially buried prey item and will take an intentional pause to prepare a lunge that is used to capture the prey (Stebbins and Kalk 1961). Their eyes are also adept at spotting predators as they have been reported to detect movement from up to 9 meters away and flee (Stebbins and Kalk 1961). Their eye positioning also lets mudskippers bask in shallow waters while still be wary of aerial threats (Tytler and Vaughn 1983). Endler (1992) suggests that a predatory/reproduction balance exists, where in if an animal is under stronger predatory pressure, they are more likely to be cryptically colored, whereas animals that not as pressured by predators are able to display vibrant color patterns in an effort to maximize mate selection success (Endler 1992). Applying this logic to the mudskippers would suggest that predation is not significant enough a pressure to require crypsis and therefor might also not be a strong enough pressure to require aerial hearing.

Our results do not support out hypothesis that *P. barbarus* would have different hearing capabilities to those of *C. carolinae*. Our results show that *P. barbarus* have almost identical hearing thresholds to those of *C. carolinae* (Figure 3). Our research also suggests that *P.*

barbarus are sensitive to substrate vibration, but that activation of their lateral line might not play as important a role as we had thought, as hearing was more sensitive during exposed substrate-borne trials. This is because having water covering the fish should result in a more direct stimulation of the lateral line. However, that the lateral line would play little role in particle motion detection in mudskippers would make sense, as mudskippers have reduced lateral lines (Ahnelt 2004).

Looking at other mudskippers, their vocalizations are remarkably similar to other soniferous gobies (Polgar et al. 2011). Mudskippers have a similar call structure, a mixture of tones and pulses, that have a main frequency near 100Hz. Of 19 gobies that were compared to the mudskipper, 18 species had calls that were composed of tones, pulses, or a mixture of both that ranged from 80-200Hz in main call frequency. The only outlier was *Gobiesoma bosc* which communicated using clicks with a call frequency of 1-5kHz. That gobies and mudskippers have similar call structures suggests that the auditory functions of mudskippers have not changed much to match their amphibious lifestyle. In fact, expanding the scope of sound production to include other soniferous benthic fish shows that mudskippers share similar call structure to darters (Family: Percidae) and sculpin (Family: Cottidae) which are not closely related to Gobiidae (Zeyl et al 2016). This suggests that life as a benthic fish exerts a stronger selective pressure on the mudskipper's auditory system than being an amphibious fish. That the Family Gobiidae (which includes *P. barbarus*) and the Family Cottidae (which includes *C. carolinae*) have similar call structures would provide solid reasoning for why *P. barbarus* did not have significantly different hearing capabilities to those of the fully aquatic fish, *C. carolinae*. While mudskippers do not possess any hearing adaptations as a result of amphibious lifestyle, that does not mean that they do not still communicate. They are known to communicate intraspecifically

by using a combination of ground-borne vibrations and visual behavior displays. In fact, mudskippers are adept in their communication. Mudskippers are suggested able to detect slight variations call structure to potentially identify other individuals of their species (Polgar et al. 2011).

Mudskippers possess several adaptations that promote their amphibious lifestyle. Some of these adaptations specifically help make their time out of the water easier, such as ways to respire without the presence of water or having eyes that increase their vision in air (Aguilar et al. 2000, Sponder and Lauder 1981). The acoustic adaptations possessed by mudskippers are not specific to life on land, but these adaptations are able to be used both aquatically and terrestrially so that they are never in an environment that inhibits their acoustic communication.

Research Significance

Amphibious animals are a fascinating group to study and are worthy of further investigation. There are many adaptations that are not only specific to amphibious animals but are taxa specific as well. Animals like turtle have a whole different set of adaptations than something like an amphibious fish. This study helps shed some light of communication techniques of the Atlantic mudskipper and gives us a better insight into amphibious acoustic communication.

FIGURES

Figure 1. The experimental testing apparatus that was used to collect auditory evoked potentials. (A.) Glass testing jar; (B.) bass shaker; (C.) respirator mouthpiece; (D.) cone speaker.

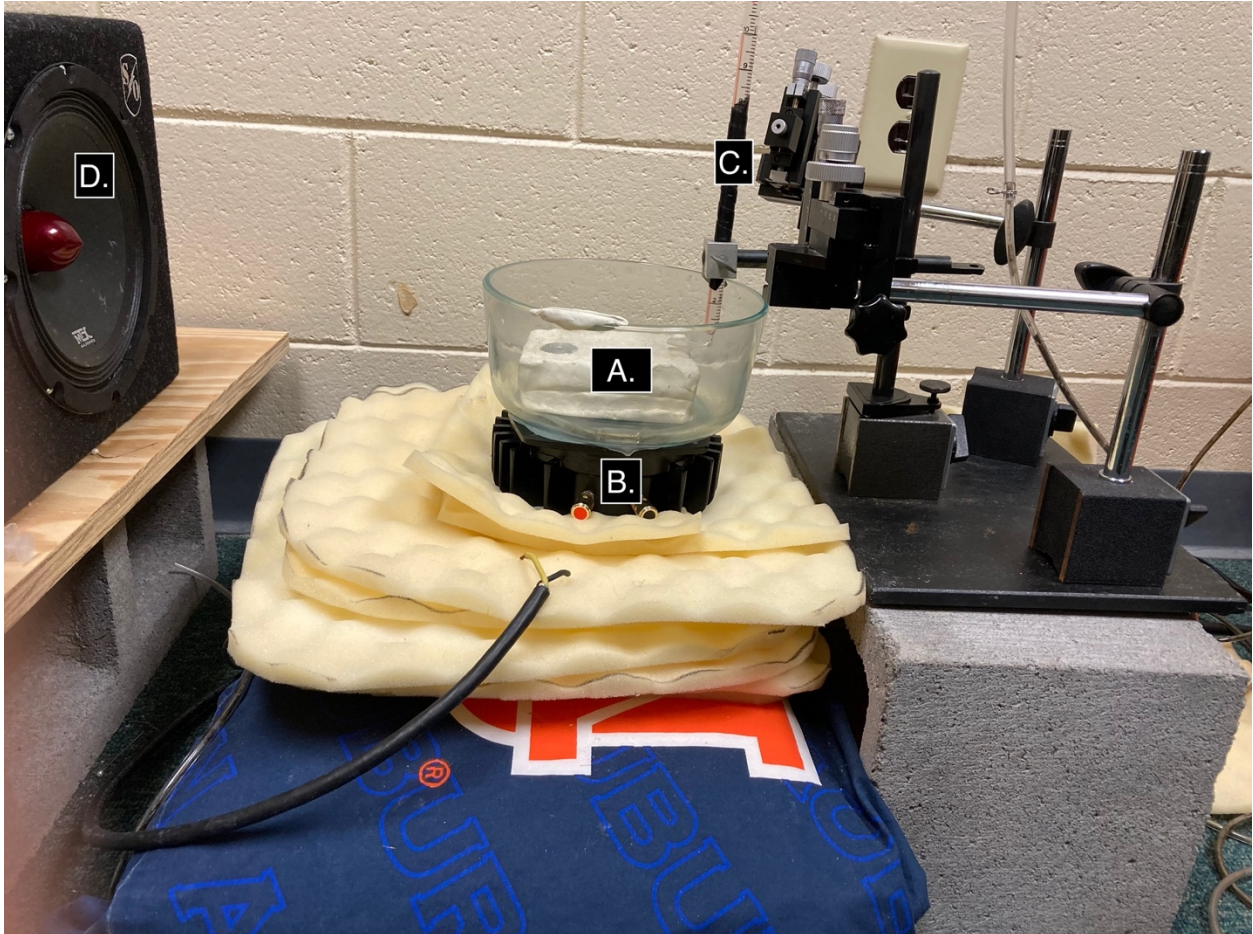
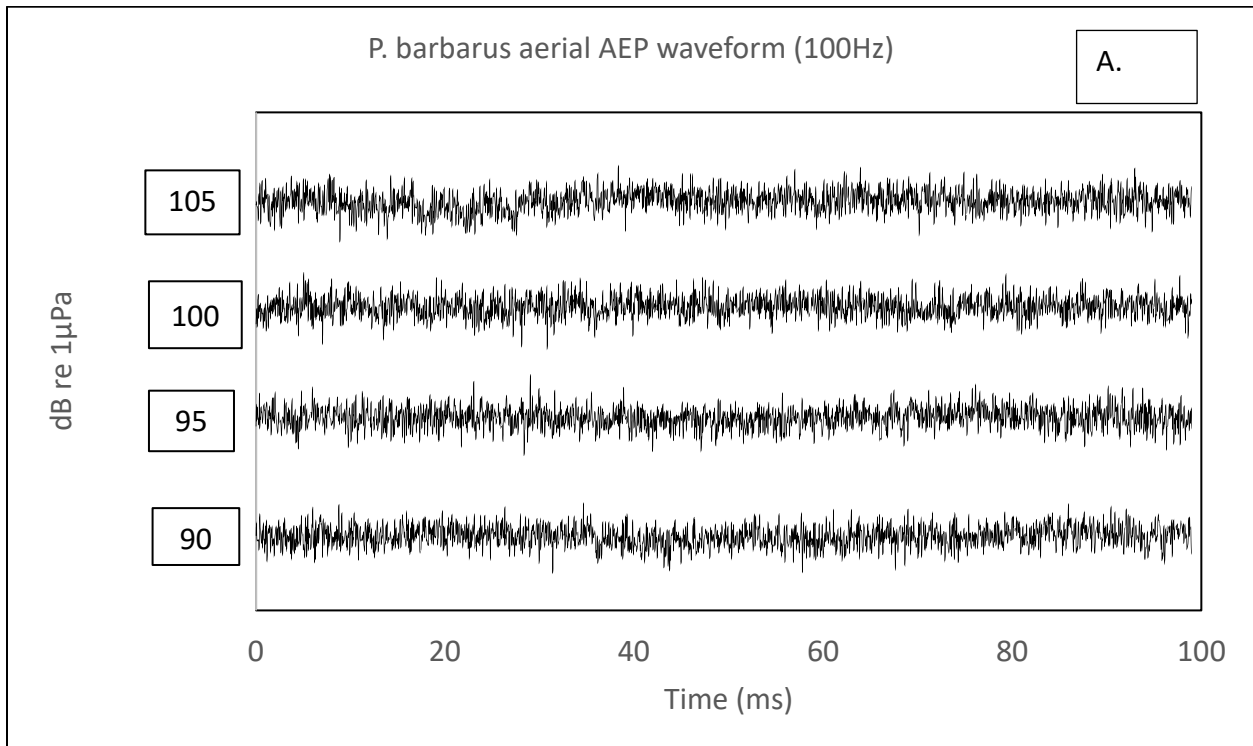
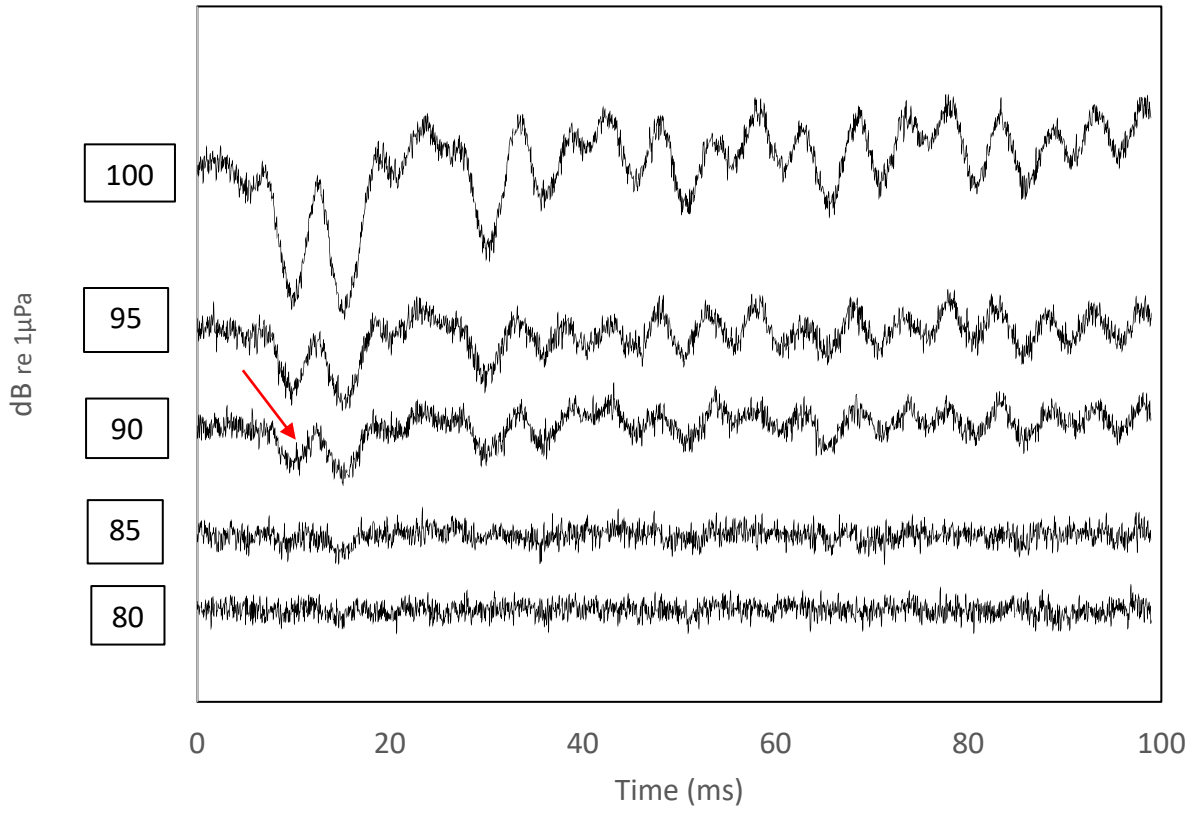


Figure 2. Representative AEP traces from an individual *P. barbarus*. Responses were collected as a result of airborne (a), exposed ground-borne (b), submerged ground-borne (c) trials at 100Hz. Arrows are included to denote the decibel level at which a threshold was reached.



P.babarus dry groundborne AEP waveforms (100Hz)

B.



P.barbarus submerged substrate-borne AEP waveform (100Hz)

C.

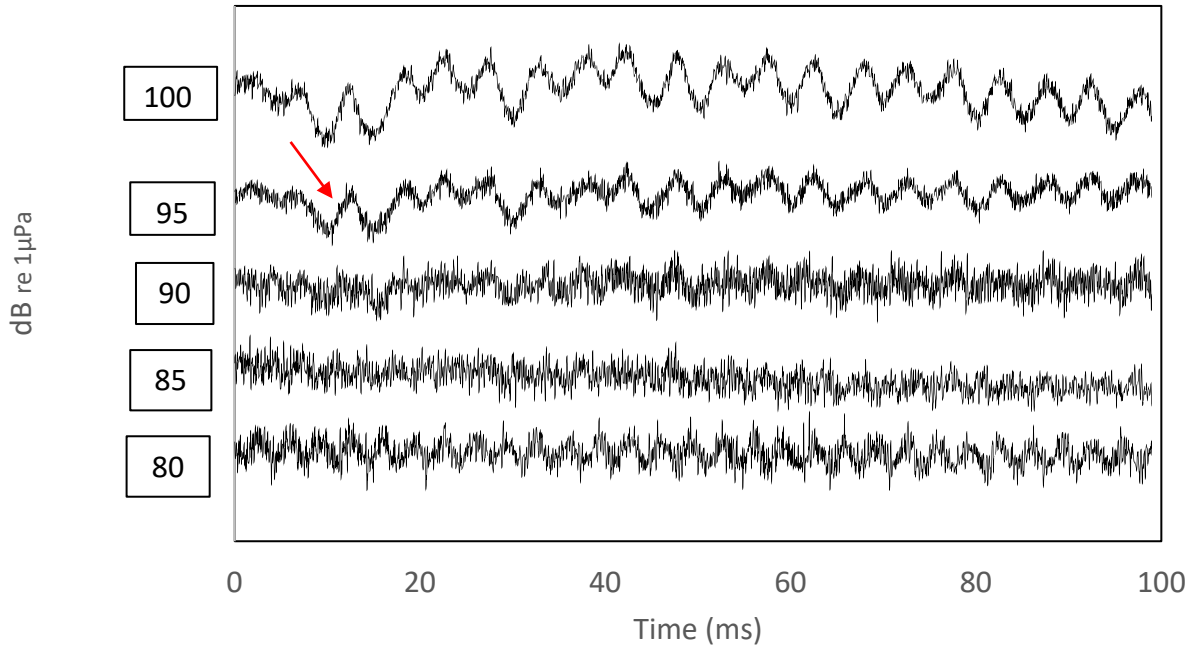
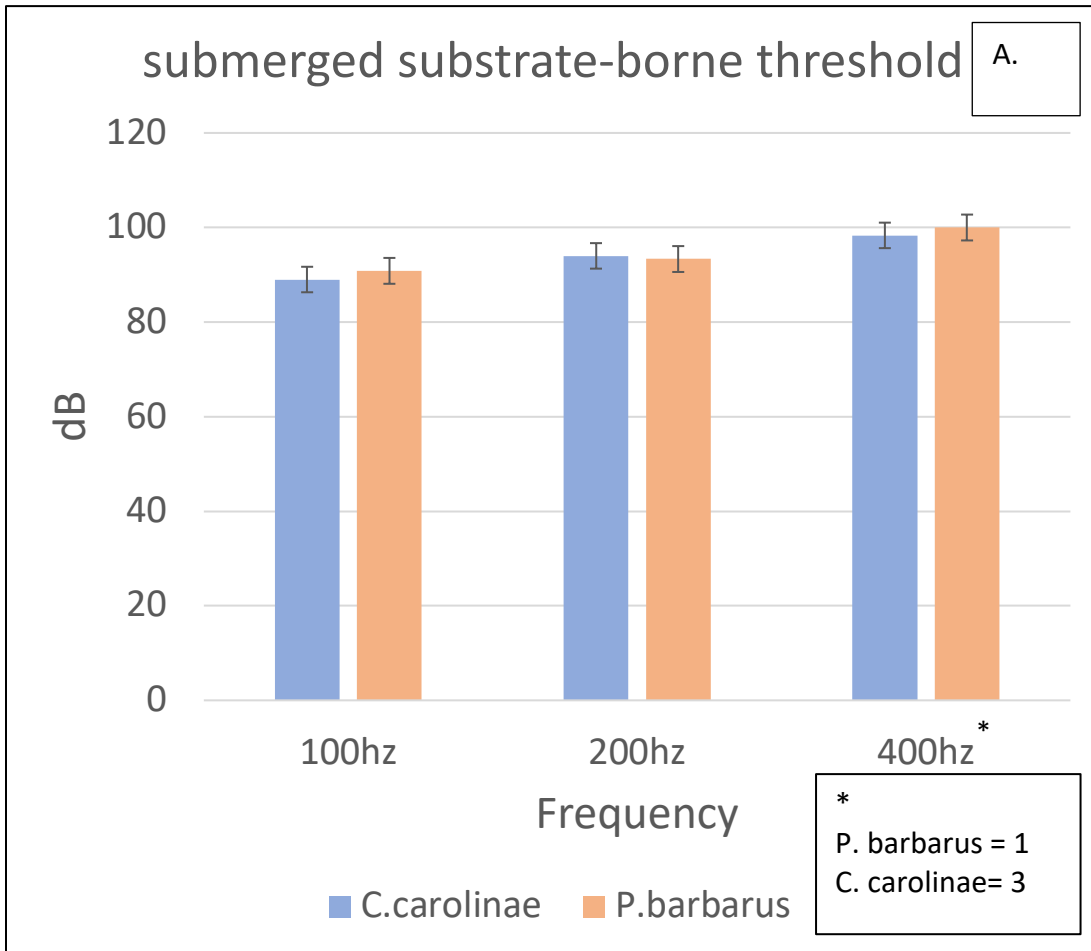
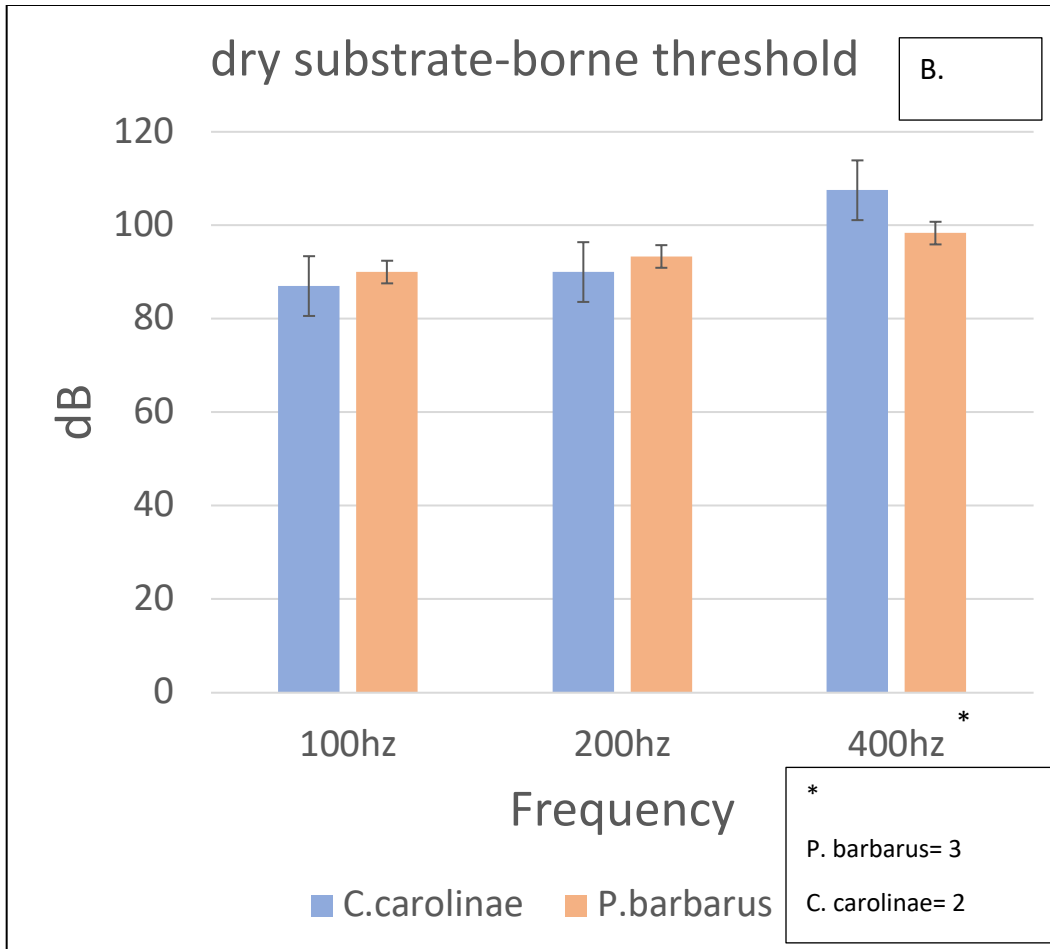


Figure 3. Audiograms comparing average threshold of *Periophthalmus barbarus* and *Cottus carolinae* (mean \pm SEM) in 100Hz, 200Hz, and 400Hz frequencies in both submerged substrate-borne trials (A) and dry substrate-borne trials (B). No aerial threshold is included because no fish registered a threshold during aerial trials. The submerged substrate-borne and exposed substrate-borne audiograms do not include 800Hz and 1600Hz thresholds because no fish registered a threshold at 800Hz or 1600Hz.





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