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JASA ARTICLE

A comparative analysis of form and function in Centrarchidae hearing ability: Does otolith variation affect auditory responsiveness?[a)]

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ABSTRACT:
There exists a wealth of knowledge on hearing ability in individual fish species, but the role of interspecific variation, and drivers behind it, remains understudied, making it difficult to understand evolutionary drivers. The current study quantified hearing thresholds for three species of sunfish in the family Centrarchidae [bluegill sunfish (*Lepomis macrochirus*), pumpkinseed sunfish (*Lepomis gibbosus*), and rock bass (*Ambloplites rupestris*)] using auditory evoked potentials and behavioral trials and saccular otolith size and hair cell density. In auditory physiological experiments, 10-ms tone bursts were played and responses monitored to measure hearing. In behavioral experiments, fish were exposed to the same tone bursts for 1 s, and changes in fish behaviors were monitored. Saccular otolith morphology and hair cell densities were also quantified. Physiological thresholds varied between species, but behavioral thresholds did not. Rock bass had larger S:O ratio (percentage of the saccular otolith surface occupied by the sulcus), but no differences in hair cell densities were found. Our study allows for a direct comparison between confamilial species, allowing a deeper understanding of sound detection abilities and possible mechanisms driving differential hearing. Using both approaches also allows future research into how these species may be impacted by increasing levels of anthropogenic noise. © *2023 Acoustical Society of America*.
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I. INTRODUCTION

Fish use sounds in their natural environments to obtain vital information about their surroundings that include biotic factors like the detection of predators and prey and location of potential mates and competitors, as well as information on abiotic factors, including currents, coastlines, and wind (Popper and Fay, 1993; Lagardère *et al.*, 1994; Fay and Popper, 2000; Amoser and Ladich, 2005; van der Sluijs *et al.*, 2011; Mickle and Higgs, 2017). To understand how fish use sound, it is vital that hearing abilities of individual species be properly quantified, and in recent years, there has been a push for combining both physiological thresholds determined through auditory evoked potential (AEP) experiments and behavioral thresholds (Popper and Hawkins, 2021) to determine hearing thresholds of fish species. By combining a physiological technique that records neural activity from the sensory hair cells, eighth cranial nerve, and brainstem auditory nuclei (Jewett, 1970; Jewett and Williston, 1971; Jacobson, 1985; Yost and Schlauch, 2001; Kenyon *et al.*, 1998; Scholik and Yan, 2002; Popper *et al.*, 2019) with changes in a fish's behavior when exposed to the same acoustic stimuli, a more comprehensive understanding of when fish are able to detect and differentiate ambient noise from potential noise stressors in their natural environments may be obtained (Popper and Hawkins, 2021), addressing the concern brought up by Popper and Hawkins (2021) that physiological thresholds only determine when the brain responds to acoustic stimuli, not when fish can hear sounds in their natural environments. Ladich and Fay (2013) also highlight a problem when comparing physiologically and behaviorally obtained hearing thresholds, as behavioral and physiological thresholds tend to differ from each other, with frequencies below 1000 Hz being approximately 10 dB lower in behavioral trials, whereas frequencies above 1000 Hz are 10 dB higher. Experiments using the AEP technique are already difficult to compare when conducted in different labs due to different methodologies, resulting in variation in thresholds (Higgs, 2002; Sisneros *et al.*, 2016), but the extreme variation in acoustic environments adds an additional obstacle when directly comparing behavioral trials and AEP trials. By combining these two techniques, we can get a better understanding of when fish are able to hear sounds in their natural environment and when anthropogenic noise sources may act as an active stressor.

How sensitive fishes are to auditory signals may be dependent on saccular variation, including the overall shape of the sagitta (Lychakov and Rebane, 1992, 2000; Schulz-Mirbach *et al.*, 2019), the area of the sulcus acousticus in relation to the otolith area (Monteiro *et al.*, 2005), the density of hair cells

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within the macula sacculi (Smith, 2016), and whether they evolved adaptations to enhance hearing abilities, such as a gas-filled body (e.g., a swim bladder) found within the fish and near the inner ear (Poggendorf, 1952) or structures to mechanically connect the swim bladder to the ear like Weberian ossicles (Popper *et al.*, 2003; Popper and Fay, 2011). The inner ears of fish species exhibit extensive diversity in morphological features, primarily in the size of their otoliths, the shape and orientation of their sensory epithelia, and the orientation patterns of their sensory hair cells (Ladich and Schulz-Mirbach, 2016). Previous studies have determined that additional hearing structures generally enhance hearing abilities (Sand and Hawkins, 1973; Popper *et al.*, 2003; Popper and Fay, 2011); however, the role otolith morphological differences play in a fish's hearing ability is less understood and often contradictory. Schulz-Mirbach and Ladich (2016) demonstrated a potential correlation between specific orientation patterns of sensory hair cells, primarily on the macula sacculi, enhancing hearing abilities when accessory hearing organs are also present (Platt and Popper, 1981), but this is not consistent across species (Schulz-Mirbach *et al.*, 2014). Contradictory findings are even found within families, as Ramcharitar *et al.* (2001) investigated variation in inner ear morphology within the family Sciaenidae, uncovering significant differences in morphology and hair cell densities between Sciaenid species. These studies highlight that fish species, even within the same family, have differences in otolith morphology; however, the role these differences play in hearing abilities between related species and the role otolith morphology may have when determining potential differences in hearing sensitives have yet to be heavily investigated.

The current study focuses on quantifying hearing thresholds for three species of sunfish in the family Centrarchidae [bluegill sunfish (*Lepomis macrochirus*), pumpkinseed sunfish (*Lepomis gibbosus*), and rock bass (*Ambloplites rupestris*)] using both AEP experiments and behavioral trials and compares any interspecific variation, or lack thereof, in their responses to differences in anatomical features of their inner ears. The Centrarchidae are a speciose family of freshwater fishes native to North America, although widely introduced in warm waters globally (Berra, 2001). As both forage fish and popular recreationally angled species, the Centrarchids are important components of freshwater ecosystems in which they occur, but it remains unclear how they might be affected by the increasing problems of anthropogenic noise of interest worldwide (Popper and Hastings, 2009; Slabbekoorn *et al.*, 2010; Whitfield and Becker, 2014). Thus, this family can be a useful model for examination of interspecific differences in hearing ability as well as important metrics for how noise may impact freshwater ecosystems once their basic hearing abilities have been determined.

II. METHODS

A. Auditory threshold experimental design

To quantify centrarchid AEP values, auditory physiology was conducted. Bluegill [*L. macrochirus*, $n = 8$, total length (TL) $= 13.125 \pm 0.1641$ cm], rock bass (*A. rupestris*, $n = 5$, TL $= 9.6 \pm 0.3883$ cm), and pumpkinseed (*L. gibbosus*, $n = 8$, TL $= 13 \pm 0.2351$ cm) were obtained from the Detroit River at Riverdance Park in Lasalle, Ontario, Canada (42.2367 °N, 83.1058 °W) through angling and seining from June 2022 to August 2022. Any species that were caught and not of interest were quickly placed back into their natural habitat. Species of interest were placed into a cooler full of river water and transferred back to the University of Windsor.

Experiments were conducted in a tank that was constructed from 1.17 m long, 25 cm diameter polyvinyl chloride pipe (5 mm thick) with an opening of 1 m × 0.15 m, filled with dechlorinated water in a sound-attenuating chamber (vocalbooths.com) to reduce the level of ambient noise (Wright *et al.*, 2005). Fish were anesthetized in 250 μl of 2-phenoxyethanol in 500 ml of dechlorinated water (0.004 M), and a constant drip of 0.002 M 2-phenoxyethanol was kept over the fishes' gills to keep the fish sedated (Higgs and Radford, 2013). Once sedated, each fish was placed onto a plastic stage held in place on a micromanipulator, wrapped in cheesecloth, and secured using a few binder clips. Silver wire electrodes (Rochester Biomedical) coated in nail polish with the tip exposed were used as recording, reference, and grounding electrodes. The recording electrode was placed under the skin along the dorsal midline, directly above the brainstem, outside of the skull. The reference electrode was placed approximately 5 mm forward, along the dorsal midline, between the nostrils. Finally, the grounding electrode was placed between the fish and the base of the fish holder. After electrode placement, the fish was lowered until the head was 5 cm underwater [Fig. 1(A)].

Tone bursts of pure tones (100–2000 Hz) were played for 10 ms in a randomized order using a dice app (Apple App Store; Dice) and emitted from a speaker (UW-30, Electro-Voice, Burnsville, MN) located in the opposite end of the tank, approximately 1 m away from the fish. 100 Hz was the lowest frequency played because it is difficult to produce clear signals below 100 Hz with standard underwater speakers and, especially in tank studies, lower frequencies are difficult to manage due to their extremely long wavelengths causing serious reverberations. Tone bursts were generated in SigGen (TDT) software and presented through a TDT System-3 evoked potential workstation. Calibration occurred at the beginning of each experiment using a Cetacean Research Technologies (Golden, CO) hydrophone (model CR1A, sensitivity = –199 dB re 1 μPa/V) at the position of the fish holder to ensure sound intensity being presented was consistent across trials. Background sound levels in the recording tank were approximately 80 dB re 1 μPa for all experiments (Fig. 2). Each frequency was played from 100 to 160 dB re 1 μPa, beginning at the lower sound level and increasing until a response was seen. Sound stimuli were presented in alternate phases (90° and 270°) with 200 presentations of each phase, and then all 400 traces were averaged for each sound level and frequency. The responses were collected and averaged in BioSig

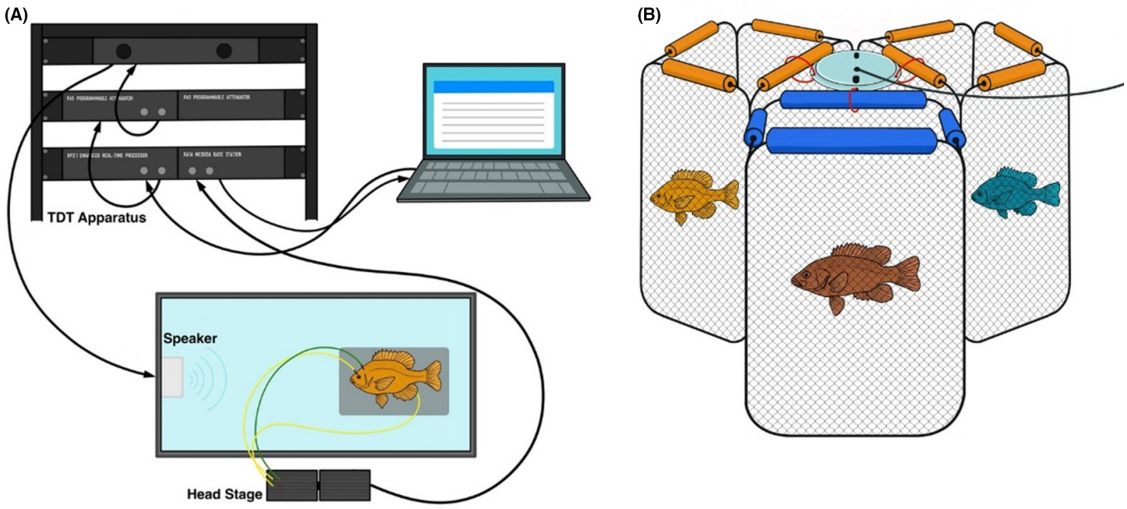

FIG. 1. (Color online) Experimental setup for (A) auditory physiology experiments, equipped with an underwater speaker, a head stage, and a Tucker-Davis Technologies (TDT) (Alachua, FL) apparatus, and (B) behavioral threshold experiments equipped with floating experimental pens and an underwater speaker in the middle of the three pens. Floating covers with GoPro HERO7 are not depicted.

through the TDT evoked potential workstation and stored for offline assessment of the auditory thresholds for each frequency.

B. Behavioral threshold experimental design

Bluegill ($n = 18$, TL $= 15.1 \pm 0.2104$ cm), rock bass ($n = 17$, TL $= 18.7 \pm 0.2966$ cm), and pumpkinseed ($n = 16$, TL $= 13 \pm 0.2770$ cm) were obtained from the Detroit River at Riverdance Park in Lasalle, Ontario, Canada (42.2367 °N, 83.1058 °W) through angling (48%) and seining (52%) during June 2022 to August 2022 and placed into a holding pen. The holding and experimental pens were made of a mesh (collapsible mesh laundry basket) material around the four perimeter sides, allowing for sound to pass through without interference [Fig. 1(B)]. Ambient sound levels varied, but total power ranged from 115 to 131 dB re 1 μPa. Any species that were caught and not of interest were quickly placed back into their natural habitat. All experiments were conducted next to a fishing dock where the water was no deeper than 1.5 m throughout the year, with an abundance of aquatic vegetation. We visually determined water clarity before starting an experiment by ensuring the fish could be seen at the bottom of the experimental pen to allow for video analysis and behavioral quantification.

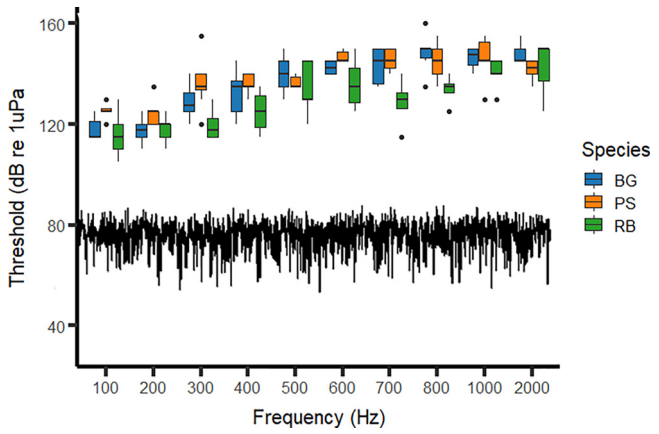

FIG. 2. (Color online) The background noise levels (black line) and physiological hearing thresholds of bluegill (BG), pumpkinseed (PS), and rock bass (RB) across frequency. Error bars are representative of mean ± standard error (s.e.) with * representing significant differences between species at a p-value of <0.05.

Three experimental pens (30 cm × 50 cm) [Fig. 1(B)] were secured together with zip ties in a triangle formation to allow for a speaker (Electro-Voice UW-30) to be attached to the top of the pens with equal distance between all of them. Styrofoam "pool noodles" were attached to the outside, top edges of the pens to allow them to float in the water. White plates with light-coloured aquarium gravel glued to them were placed at the bottom of the pens to allow for an appropriate amount of contrast for a GoPro (HERO7 Silver), secured to the top of the pens with the lens underwater, to pick up different behaviors of the fish if they were sitting at the bottom of the pen. The speaker was connected to an amplifier (SA300, Scosche, Oxnard, CA) and an MP3 player (Apple iPod Touch) with the different tones played to the fish once throughout the trial. The tones played for a total of 1 s with the frequencies of the tones ranging from 100 to 2000 Hz with each of the frequencies played at six different intensities ranging from 95 to 165 dB. 100 Hz was the lowest frequency played because it is difficult to produce clear signals below 100 Hz with standard underwater speakers. Sound levels were calibrated at the midpoint of the experimental pen with a Cetacean Research Technologies hydrophone (model number CR1A, sensitivity $= -199$ dB re 1 μPa/V) to ensure sound intensity being presented was consistent across trials. Due to the small size of the pen, sound levels were consistent throughout the pen.

Fish were removed from the holding pen, placed into the experimental pen, and given a 10-min acclimation period to reduce any potential handling stress. Once the experiment began, fish were given a 1-min control period

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with no sound played, followed by the 1-s tone, and finally another control period. The second control period varied from 1 to 2 min depending on the likelihood a threshold was hit. If there was no change in the fish's behavior, they were given 1 min, and if there was a behavioral response, the fish were given 2 min to ensure they had ample time to reduce their overall stress levels to the played sound file. The order in which the frequencies were played was randomized using a dice app (Apple App Store; Dice) to avoid any internal bias, but the order of the levels was always played from quietest to loudest to reduce habituation. Experiments took approximately 90 min to complete, including a final 2-min control after the final tone was played. Following the final 2-min control, the fish were removed from their experimental pen, their length was measured to the nearest half millimeter, their dorsal fins were fin clipped, and they were released back to their natural environment. Fin clipping allowed for the fish to be returned to their natural habitat and ensured that the same fish would not be used in an experiment conducted later in the field season. Behavioral thresholds were determined post-processing and deemed to have been reached when a fish displayed a sudden change in their movement patterns (e.g., onset of rapid swimming, an increase in fin beats, a rapid turn, and/or freezing at acoustic stimulus onset) to account for individual responses to sudden acoustic stimuli exposure. Observers were not blind to treatment while determining whether a behavioral threshold was reached, due to difficulties finding where 1-s sound exposure occurred in a 90-min experimental trial.

C. Otolith characterization and hair cell counting

A total of 60 saccular otoliths (30 paired left and right sagittae) were extracted from bluegill (TL = 15 ± 0.7089 cm), rock bass (TL = 14.4 ± 0.9130 cm), and pumpkinseed (TL = 13.8 ± 0.8474 cm) ($n = 10$ for each). Specimens were obtained from the Detroit River at Riverdance Park in Lasalle, Ontario, Canada (42.2367 °N, 83.1058 °W) through angling and seining during July 2021 to October 2021. The length of each specimen was measured to the nearest half millimeter, after which they were euthanized through an overdose of an anesthetic (clove oil or 2-phenoxyethanol). Specimens were stored in buffered 4% paraformaldehyde until the final dissection was completed in mid-February 2022. The saccular epithelia and otoliths were dissected from the head, stored in 4% paraformaldehyde, and then prepared for staining.

A Leica (Wetzlar, Germany) S6D stereo microscope with an attached QICAM Fast 1394 digital camera was used to image each sagitta. The software Northern Eclipse version 8.0 was then used to record the otolith area (mm^2) and the sulcus (a structure found on the medial side of the otolith; Song *et al.*, 2019) length (mm) and area (mm^2). These measurements were used to calculate the percentage of the otolith surface occupied by the sulcus [sulcus to otolith (S:O) ratio; Osman *et al.*, 2021; D'Iglio *et al.*, 2022]. To visualize hair cells, a total of 18 saccular macula [bluegill (TL = 14.5 ± 0.7746 cm), rock bass (TL = 13.6 ± 0.4362 cm), and pumpkinseed (TL = 15.8 ± 0.7159 cm, $n = 6$ for each)] were stained using 12.5 μl of fluorescent green phalloidin mixed with 200 μl of phosphate buffer (Higgs *et al.*, 2002), mounted, and coverslipped for imaging using fluorescence microscopy (Leica M205FA stereo microscope). To quantify hair cell density, images were imported into Adobe Photoshop (version 3.0; Adobe Systems, San Jose, CA) to create three identical boxes of 225 μm^2 in size representing 19% of the total saccular area (Higgs *et al.*, 2002). Ciliary bundles were counted in three regions along the anterior, middle, and posterior saccule using a magnified view of the epithelium and within each box were then counted using Image 224J software (National Institutes of Health).

D. Statistical analysis

Threshold data for AEP and behavioral experiments are presented in units of sound pressure rather than particle motion as it remains difficult to obtain accurate measures of particle motion in the field (Nedelec *et al.*, 2016) as a main focus of the paper was to make comparisons between species in lab and field measurements. All statistical analyses were performed using SPSS statistics version 28.0.1.1. Before subjecting the auditory threshold and behavioral threshold data to any testing, fish that never showed a response or were out of camera view were removed from the data set. All data were determined to be normal due to their distribution on Q-Q plots. Body size for physiological threshold data was subjected to a one-way analysis of variance (ANOVA) to determine whether there was a significant difference between the species, and due to a significant difference ($F_{2,166} = 43.481$, $p < 0.001$), a two-way analysis of covariance (ANCOVA) with species and frequency as the main effects, thresholds as the dependent variable, and body size as the covariate was conducted, followed by a Bonferroni *post hoc* test. Body size for behavioral threshold data was subjected to a one-way ANOVA to determine whether there was a significant difference between the species, and due to a significant difference ($F_{2,433} = 1105.987$, $p < 0.001$), two-way ANCOVA with species and frequency as the main effects, thresholds as the dependent variable, and body size as the covariate was conducted, followed by a Bonferroni *post hoc* test. Both S:O ratio and hair cell densities were subjected to a one-way ANOVA, followed by a Tukey's honest significant difference (HSD) test if a significant result was present. There was no effect of body size on S:O ratio ($F_{2,27} = 0.526$, $p = 0.597$) or hair cell densities ($F_{2,15} = 2.724$, $p = 0.098$).

III. RESULTS

A. Physiological threshold

Overall, there was a significant difference in physiological thresholds between species ($F_{2,138} = 8.667$, $p < 0.001$) with rock bass significantly more sensitive than bluegill ($p = 0.017$) and pumpkinseed ($p < 0.001$), but there was no significant difference in sensitivities between bluegill and pumpkinseed ($p = 0.344$; Fig. 2). Especially at higher frequencies, rock bass were 6–10 dB more sensitive than the other two species. There was an effect of frequency on threshold ($F_{9,138} = 31.799$,

$p < 0.001$) with all three species having highest sensitivity to frequencies of 100 and 200 Hz and decreasing sensitivity as frequency increased (Fig. 2, Table I). There was no significant interaction effect between species and frequencies ($F_{18\,138} = 1.301$, $p = 0.196$), and there was a significant effect of size as a covariate ($F_{1,138} = 7.718$, $p = 0.006$).

B. Behavioral thresholds

Overall, there were no significant differences in behavioral thresholds between the species ($F_{2,405} = 120.449$, $p = 0.062$; Fig. 3); however, there was a significant difference between the frequencies ($F_{9,405} = 263.655$, $p < 0.001$) with all three species having their best frequencies at 100 and 200 Hz (Table I). Across frequencies, behavioral thresholds ranged from 99 to 150 dB re 1 μPa, but within a frequency, species thresholds were all within 1–2 dB of each other (Fig. 3). There was a significant interaction between species and frequency ($F_{18,405} = 2.059$, $p = 0.007$) but no significant size effect ($F_{1,405} = 0.645$, $p = 0.422$). When comparing physiological and behavioral thresholds, behavioral thresholds tended to be lower than physiological thresholds in all three species, with behavioral thresholds being up to 15 dB lower than physiological estimates at 100 and 200 Hz but then converging in values at higher frequencies (Fig. 4).

TABLE I. Hearing thresholds for bluegill (BG), rock bass (RB), and pumpkinseed (PS) at each of the ten frequencies fish were exposed to during auditory physiology experiments re 1 μPa.

Frequency (Hz)	Species	Total number of trials	Threshold (dB re 1 μPa)
Physiological thresholds			
100	BG	8	117.394 ± 2.439
	RB	5	117.679 ± 3.126
	PS	7	124.980 ± 2.436
200	BG	8	116.769 ± 2.439
	RB	5	119.679 ± 3.126
	PS	7	123.730 ± 2.436
300	BG	8	128.644 ± 2.439
	RB	4	122.260 ± 3.524
	PS	6	135.605 ± 2.614
400	BG	7	131.547 ± 2.601
	RB	4	127.260 ± 3.524
	PS	6	135.491 ± 2.614
500	BG	5	139.423 ± 3.074
	RB	5	135.679 ± 3.126
	PS	5	136.420 ± 2.801
600	BG	6	141.741 ± 2.813
	RB	4	138.510 ± 3.524
	PS	5	146.420 ± 2.801
700	BG	6	142.574 ± 2.813
	RB	4	131.010 ± 3.524
	PS	6	145.118 ± 2.601
800	BG	6	147.574 ± 2.813
	RB	4	136.010 ± 3.524
	PS	2	144.697 ± 3.961
1000	BG	4	145.263 ± 3.447
	RB	5	141.679 ± 3.126
	PS	5	145.074 ± 2.813
2000	BG	3	147.802 ± 3.802
	RB	3	141.933 ± 3.961
	PS	5	141.477 ± 2.801
Behavioral thresholds			
100	BG	17	99.12 ± 1.590
	RB	11	103.18 ± 2.016
	PS	14	100.67 ± 1.709
200	BG	17	100.29 ± 1.591
	RB	13	102.31 ± 1.853
	PS	13	100.71 ± 1.771
300	BG	17	117.06 ± 1.590
	RB	12	114.58 ± 1.909
	PS	16	117.94 ± 1.661
400	BG	17	120.29 ± 1.591
	RB	12	123.33 ± 1.922
	PS	13	119.29 ± 1.764
500	BG	16	127.19 ± 1.640
	RB	14	125.36 ± 1.779
	PS	14	122.67 ± 1.709
600	BG	17	128.82 ± 1.590
	RB	13	119.17 ± 1.930
	PS	12	123.46 ± 1.831
700	BG	17	134.12 ± 1.591
	RB	12	138.75 ± 1.920
	PS	15	131.25 ± 1.658
800	BG	16	139.69 ± 1.640
	RB	15	138.85 ± 1.853
	PS	13	137.86 ± 1.773
1000	BG	16	144.69 ± 1.629
	RB	14	144.64 ± 1.772
	PS	14	140.67 ± 1.717
2000	BG	15	149.33 ± 1.693
	RB	12	146.82 ± 2.016
	PS	13	150.00 ± 1.771

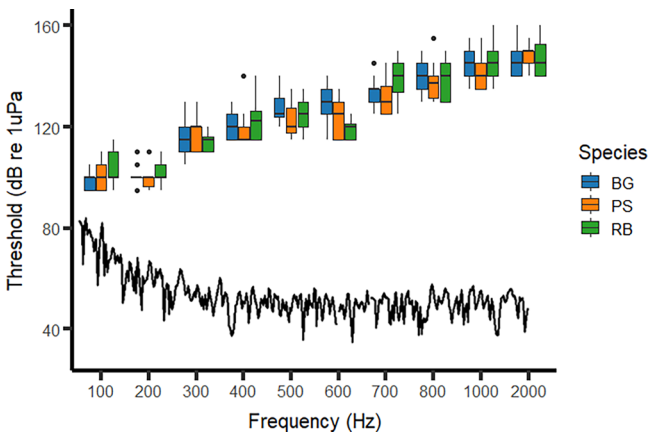

FIG. 3. (Color online) The background noise levels (black line) and behavioral response thresholds of bluegill (BG), pumpkinseed (PS), and rock bass (RB) across frequency. Error bars are representative of mean ± s.e. with * representing significant differences between species at a p-value of <0.05.

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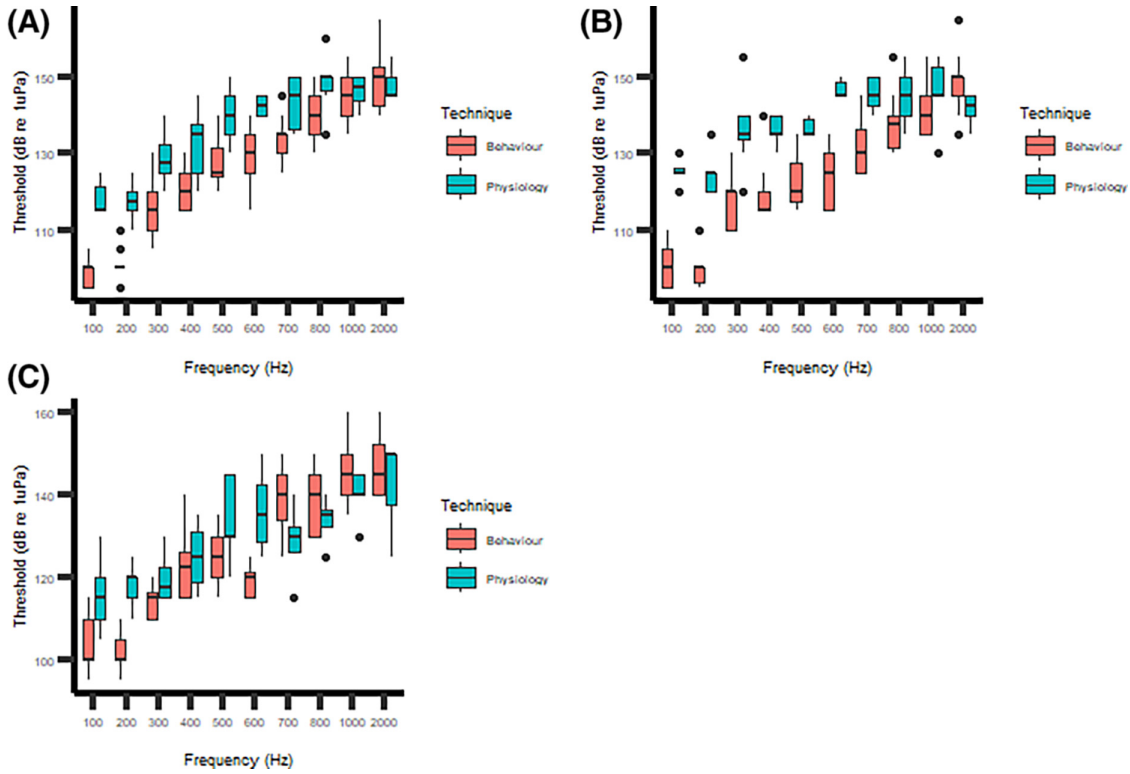

FIG. 4. (Color online) Comparison of hearing and behavioral thresholds for (A) bluegill, (B) pumpkinseed, and (C) rock bass at each of the ten frequencies fish were exposed to during both experiment types. Error bars are representative of mean ± s.e.

C. S:O ratio results

Overall, there was a significant difference when comparing the S:O ratio between rock bass and the two *Lepomis* species [$F_{2,27} = 9.167$, $p < 0.001$; Figs. 5(A) and 6], and when exploring the results of the Tukey *post hoc* test, rock bass had a significantly larger S:O ratio when compared to both bluegill ($p = 0.004$) and pumpkinseed ($p = 0.002$). The rock bass sulcus took up approximately 25% of the saccular otolith, but in the other two species, the ratio was closer to 20%. There was no significant difference in S:O ratio between bluegill and pumpkinseed ($p = 0.945$), with each sulcus encompassing approximately 20% of the saccular otolith [Figs. 5(A) and 6].

D. Hair cell density results

Overall, there was no significant difference when comparing hair cell densities between species [$F_{2,15} = 1.298$, $p = 0.302$; Fig. 5(B)]. While rock bass did tend to have a lower density of saccular hair cells, all three species had between 450 and 500 hair cells per 225 μm^2 box with no statistically discernable difference between them.

IV. DISCUSSION

A. Physiological thresholds

Based on the AEP data, all three species are most sensitive to lower frequency sounds as would be expected from

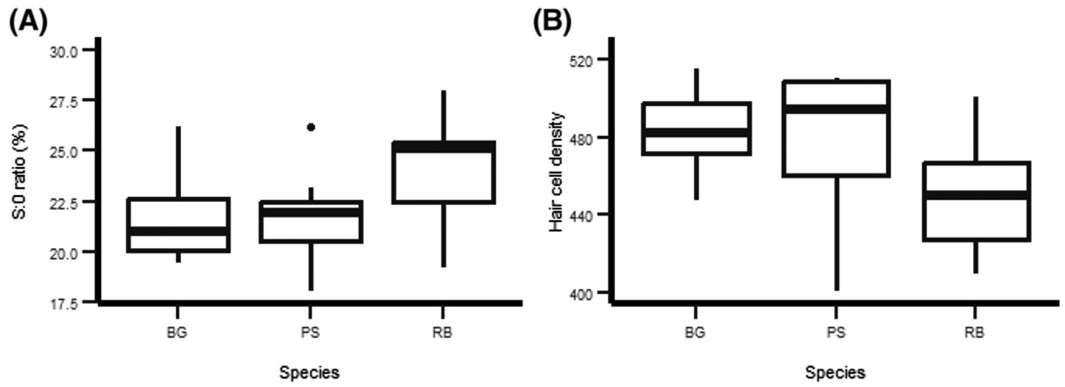

FIG. 5. The (A) S:O ratio and (B) hair cell densities in number of hair cells per 225 μm^2 box for bluegill (BG), pumpkinseed (PS), and rock bass (RB). Error bars are representative of mean ± s.e. with * representing significant differences between species at a p-value of <0.05.

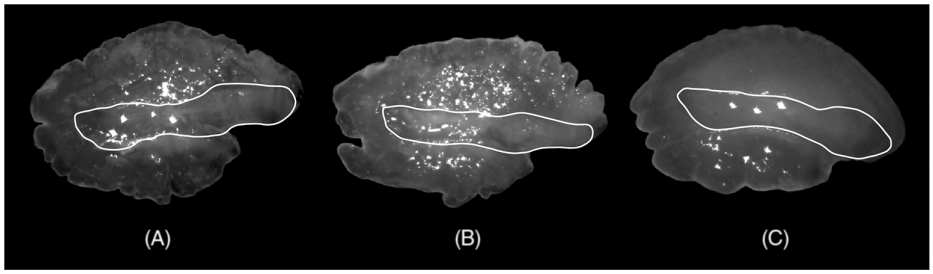

FIG. 6. The saccular otoliths of (A) bluegill, (B) pumpkinseed, and (C) rock bass with the auditory sulcus outlined with a white line in each otolith.

species without known specializations for enhanced pressure detection. While this is the first report for rock bass auditory thresholds, our results for bluegill and pumpkinseed are higher than the thresholds determined in the previous two studies (Scholik and Yan, 2002; Wysocki and Ladich, 2005). It is important to note that although the bluegill and pumpkinseed physiological thresholds were previously determined, these experiments were conducted in two different labs (Scholik and Yan, 2002; Wysocki and Ladich, 2005). The problem that arises when comparing thresholds between labs is that it is difficult to directly compare results due to differences in methods, techniques, and experimental environments resulting in differences in hearing thresholds for the same species (Higgs, 2002). For example, when comparing general methods between the current study, Scholik and Yan (2002) and Wysocki and Ladic 2005 used Flaxedil to reduce myogenic noise, had the speaker playing the acoustic stimuli suspended in the air, and had the top of the fish's head out of the water where the recording electrode was placed. Wysocki and Ladic 2005 was also the only study to have sand in the bottom of their experimental tank, and all three of our studies had very different sized experimental tanks and obtained our fish from different sources (Scholik and Yan from a hatchery, Wydsocki and Ladich from a pet store, and ours wild caught). With these major differences, it is difficult to compare physiological thresholds between labs; however, the current study allows for a direct comparison of these thresholds between the three species as the methods are consistent.

While none of the species tested in the current study have connections between swim bladders and inner ears, the rock bass did have a higher S:O ratio, which may explain their lower AEP thresholds at higher frequencies. Determining the S:O ratio allows for an approximation of the size of the macula sacculi in relation to the otolith, with the model of hearing of Gauldie (1988) stating that a larger relative macula size correlates to a higher hearing sensitivity due to a greater shearing force inflicted by the otolith on the hair cells. Arellano *et al.* (1995) found variation in the S:O ratios of two Gobiidae congeners and showed the species with the larger ratio was more sensitive to acoustic stimuli. While more interspecific comparisons need to be done along these lines, this does offer intriguing evidence that S:O ratio could be a strong predictor of at least physiological sound detection abilities.

Because S:O work has not been completed for centrarchid fishes, we can only discuss species in families where S:O ratio has been heavily researched. For example, Ramcharitar *et al.* (2006) focused on determining hearing thresholds for two sciaenid fishes, the weakfish (*Cynoscion regalis*) and the spot (*Leiostomus xanthurus*), with differences in their inner ear morphologies and additional hearing structures. The weakfish had been determined to have additional hearing structures that ultimately enhanced their hearing abilities; however, there were no significant differences when comparing physiological thresholds between 200 and 700 Hz with the spot, who lack these additional structures, suggesting that additional hearing structures were not the factor that influenced physiological thresholds in these two sciaenid species but instead variation in otolith morphology. Weakfish had more stereocilia per hair bundle than spot, which may play a role in expanding their frequency detection range, but more research is needed on the role of stereocilia density across fishes. Ramcharitar *et al.* (2006) and the current study, therefore, both highlight that otolith morphology may play a critical role in determining physiological thresholds and must be taken into consideration when comparing species in the same family.

B. Behavioral thresholds

Despite the differences in AEP detection thresholds, there were no statistical interspecific differences in behavioral responses to sound. Although no statistical differences were found, these behavioral responses can still provide useful information on how a fish interacts with their environment and can be used to predict potential responses to acoustic stimuli, which is vital given the rise in underwater anthropogenic noise (Popper and Hastings, 2009; Slabbekoorn *et al.*, 2010; Whitfield and Becker, 2014). In previous research, it has been common to use classical, operant, or reward conditioning to determine when a fish can hear different sounds; however, conditioning requires fish to be trained to communicate with the researcher when they are able to detect the acoustic stimuli they are being presented with (Popper and Hawkins, 2021). The current study allows for a different approach as fish did not have to be trained, and instead, we examined their natural behaviors in which fish would also have to discriminate between the acoustic stimuli they are being experimentally exposed to, from sounds in their natural environment. By conducting behavioral threshold experiments in a fish's natural environment, it can be determined when sounds are causing changes to their behavior when other natural sounds are present.

Although the acoustic environment varied between physiological and behavior threshold experiments, as physiological thresholds were determined in a tank and behavioral thresholds were determined in the field, both

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approaches can provide useful information on a fish's hearing abilities, and both field and laboratory approaches can achieve similar overall results (Pieniazek *et al.*, 2020). Ladich and Fay (2013) found physiological thresholds are on average 10 dB higher than behavioral thresholds, and this matches the pattern seen in the current study, although background noise levels were higher in the field setting for the behavioral trials. Variation between physiological and behavioral thresholds can exist for multiple reasons, with one of them being variation in stimuli duration. In the current study, fish in auditory physiology experiments were exposed to the acoustic stimuli for 10 ms, whereas fish in behavioral experiments were exposed to acoustic stimuli for 1 s. However, there are conflicting conclusions on the role stimuli duration plays in threshold determination, as Hawkins (1981) found behavioral thresholds to be higher when signal duration decreases in Atlantic cod (*Gadus morhua*), with Fay and Coombs (1983) and Garabon and Higgs (2017) coming to the same conclusion when examining thresholds in goldfish, but Popper (1972) found there to be no effect when examining goldfish thresholds. All fish during our experiments being exposed to the same acoustic stimuli in the same environments allows for a direct comparison between experiment types; however, caution is always advised when comparing behavioral and hearing thresholds.

One possible limitation of the current study is the differences in the average size of rock bass between auditory physiology and behavioral trials, but we do not feel this biased the results herein for two reasons. First, there was no size difference within the other two species between methodologies, and they showed the same increased sensitivity in behavioral trials as did the rock bass. Second, there was no effect of size as a covariate in behavioral trials, and once fish leave the larval stage, there is no effect of size on AEP results in other species (Higgs *et al.*, 2002). Fish in our experiments were wild caught, and individuals who were previously used in trials were fin clipped and released back into their natural environment. To ensure experiments were conducted around the same time of day, using individuals from the same environment not previously used in other trials, we were limited by the fish we were able to catch. This limitation and the time of year each experiment type was conducted led to differences in the average size between experiment types. There may be a size effect on S:O ratio, although the results are mixed (Lombarte 1992; Arellano *et al.*, 1995; Montanini *et al.*, 2015; Taylor *et al.*, 2020), but the fish used for morphological analysis were of a much smaller size range in the current study.

This is the first study to quantify auditory thresholds and behavior thresholds in one lab with multiple species in the family Centrarchidae. We present all data in terms of pressure rather than particle motion because there remains no tractable way to measure particle motion in the field for fish studies that is not cost-prohibitive (Nedelec *et al.*, 2016), and our goal was to compare threshold between techniques and between species, so the absolute intensity metric is less important than the patterns. In addition, regulators are more likely to measure sound intensity in terms of pressure units than particle motion/acceleration. Our findings suggest that fish within the same family who have the same general hearing structures cannot be assumed to have the same hearing thresholds, as inner ear morphology may vary, causing variation in hearing sensitivities. In future studies, there should be a push for multimodal experimental setups when attempting to determine when fish can hear sounds, as physiological hearing thresholds provide the bandwidth in which fish can hear, behavioral thresholds provide when the fish is affected by the acoustic stimuli, and otolith characterization provides insight for why variation exists. Fish that lack additional hearing structures should also be focused on, as it cannot be assumed they have the same hearing abilities and, therefore, are affected in the same way when exposed to the same sounds.

Overall, these three species of fish have different physiological thresholds, the same general behavioral thresholds, different S:O ratios, and the same general hair cell densities. Conducting all three types of experiments in the same lab allows for a direct comparison between the three species, which is something that has never been done before in this family. All three species are commonly found together forming natural communities; therefore, the results of our study allow for a comprehensive understanding of when these species can be affected by acoustic stimuli present in their natural environments. To fully understand when fish are able to hear, distinguish, and be affected by noises in their environments, hearing and behavioral thresholds must be determined, and otolith characterization is required to understand any differences that lie between species when comparing their respective thresholds.

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