

Intra- and Intersexual swim bladder dimorphisms in the plainfin midshipman fish (*Porichthys notatus*): Implications of swim bladder proximity to the inner ear for sound pressure detection

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Abstract

The plainfin midshipman fish, *Porichthys notatus*, is a nocturnal marine teleost that uses social acoustic signals for communication during the breeding season. Nesting type I males produce multiharmonic advertisement calls by contracting their swim bladder sonic muscles to attract females for courtship and spawning while subsequently attracting cuckholding type II males. Here, we report intra- and intersexual dimorphisms of the swim bladder in a vocal teleost fish and detail the swim bladder dimorphisms in the three sexual phenotypes (females, type I and II males) of plainfin midshipman fish. Micro-computerized tomography revealed that females and type II males have prominent, horn-like rostral swim bladder extensions that project toward the inner ear end organs (sacculle, lagena, and utricle). The rostral swim bladder extensions were longer, and the distance between these swim bladder extensions and each inner-ear end organ type was significantly shorter in both females and type II males compared to that in type I males. Our results revealed that the normalized swim bladder length of females and type II males was longer than that in type I males while there was no difference in normalized swim bladder width among the three sexual phenotypes. We predict that these intrasexual and intersexual differences in swim bladder morphology among midshipman sexual phenotypes will afford greater sound pressure sensitivity and higher frequency detection in females and type II males and facilitate the detection and localization of conspecifics in shallow water environments, like those in which midshipman breed and nest.

KEYWORDS

communication, gas bladder, hearing, lagena, sacculle

1 | INTRODUCTION

Teleost fishes have swim bladders that serve multiple primary functions including operating as an oxygen reservoir, regulating buoyancy, and maintaining hydrostatic position in the water column (Blaxter, Denton, & Gray, 1979; Harden Jones & Marshall 1953; Pelster, 2011). In some cases, a secondary swim bladder function has evolved that enables these species of fish to produce sound for acoustic communication (Fine & Parmentier, 2015; Kasumyan, 2008; Parmentier & Diogo, 2006). In grunts (Haemulidae) and clownfishes (Pomacentridae), the swim bladder acts as an acoustic amplifier to increase the sound level produced by the stridulation of hard skeletal parts including vibrations of the rib cage driven by jaw snapping (Bertucci, Ruppe, Van Wassenbergh, Compere, & Parmentier, 2014; Colleye, Nakamura, Frédéric, & Parmentier, 2012; Parmentier et al., 2007). In toadfishes and midshipman (Batrachoididae), sound is produced predominantly by reproductive males that have enlarged sonic muscles attached to their swim bladders and when contracted vibrate the bladder to yield high sound level acoustic signals for social communication (Bass & McKibben, 2003; Fine, Burns, & Harris, 1990; Fine, Bernard, & Harris, 1993; Fine, Malloy, King, Mitchell, & Cameron, 2001; Fine & Parmentier, 2015).

As an acoustic organ, the swim bladder in many species of fish can also aid in the reception of acoustic signals. All fish are thought to be able to detect the particle motion component of sound using their inner-ear otolithic end organs as biological accelerometers that respond directly to the displacement of the fish by particle motion (De Vries, 1950; Fay & Popper, 1980; Sisneros & Rogers, 2016). In some species of more recently derived teleost fishes, the detection of sound pressure has evolved through the use of specialized morphological adaptations that permit pressure-induced vibrations of the swim bladder to be transduced to the inner ear for the detection of sound pressure. These “pressure sensitive” teleosts have evolved either skeletal adaptations known as Weberian ossicles (e.g., Otophysans) that connect the anterior part of the swim bladder to the inner ear, or possess gas-filled vesicles that are in close proximity to the inner ear to enhance sound pressure sensitivity (e.g., herring, Clupeidae and squirrelfishes, Holocentridae; Allen, Blaxter, & Denton, 1976; Blaxter, Denton, & Gray, 1981; Braun & Grande, 2008; Coombs & Popper, 1979; O’Connell, 1955; Popper & Coombs, 1980). The detection of sound pressure is also thought to be important for sound source localization by fishes. Current models for sound source localization depend on the detection and processing of both sound pressure and acoustic particle motion (Hawkins, 1986; Schuijff, 1981; Sisneros & Rogers, 2016; Zeddies, Fay, & Sisneros, 2011). Recent behavioral studies of the plainfin midshipman fish (*Porichthys notatus*) have suggested that the swim bladder is necessary for both acoustic pressure detection and near-field sound source localization (Coffin et al., 2014).

The plainfin midshipman has become the focus of recent sound localization studies because gravid females exhibit robust phonotaxis and localization of simulated male advertisement calls during the breeding season (Coffin et al., 2014; McKibben & Bass, 1998; Zeddies, Fay, Alderks, Shaub, & Sisneros, 2010; Zeddies et al., 2012). During the

late spring and summer, nesting or type I male plainfin midshipman attract females by producing a multiharmonic advertisement call known as a “hum.” Midshipman fish also produce two other vocalizations known as “grunts” and “growls” used in agonistic social interactions. As in other batrachoidid fishes, vocal signals are produced by the contraction of the sonic muscles attached to the swim bladder. The hum contains a fundamental frequency (80–102 Hz; varies with temperature) with multiple harmonics that range up to 1 kHz (Bass et al. 1999). Females rely on their auditory sense to detect and locate vocalizing mates. Previous studies have shown that females exhibit seasonal changes in physiological auditory sensitivity and morphological changes in saccular hair cell density that enhance their ability to detect and locate males that “sing” in their nests (Coffin, Mohr, & Sisneros, 2012; Rohmann, Fergus, & Bass, 2013; Sisneros, 2009; Sisneros, Forlano, Deitcher, & Bass, 2004). Once in the nest, females lay their eggs while type I males externally fertilize the eggs and then guard them throughout development. The advertisement call not only attracts females but also type II males or “sneakers” that use an alternative mating strategy to obtain fertilizations from type I males actively courting females in the nests (Brantley & Bass, 1994). Adult type II males are significantly smaller than nesting type I males and these “sneaker” males do not invest energy in building or guarding nests nor in parental care, but instead invest in the development of their testes, which can be up to 20% of their body mass (BM) for increased sperm competition (compared to the testes of type I males which are only 1%–3% of their BM; Brantley, Wingfield, & Bass, 1993).

While previous gross morphological differences in swim bladder sexual dimorphisms in the plainfin midshipman have been reported earlier by Bass and Marchaterre (1989), we now provide a more detailed examination of swim bladder sexual dimorphisms among the three midshipman sexual phenotypes: females, type I and type II males. We provide evidence for both intrasexual and intersexual dimorphisms of the swim bladder and differential distance measurements between the swim bladder and inner ear in the plainfin midshipman and interpret our findings as they relate to possible morphological adaptations for the detection of sound pressure and its implications for acoustic communication in this species.

2 | MATERIALS AND METHODS

2.1 | Animals

Adult plainfin midshipman, *P. notatus* (Girard, 1854), were collected by hand at low tide during the spring breeding season near Marshall in Tomales Bay, CA. Animals used for computerized tomography (CT) were housed in flow-through seawater aquaria at the Marine Resources Center at the Marine Biological Laboratory in Woods Hole, MA where they were maintained at 13°C–14°C on a 14:10 hr light cycle and fed live mysid shrimp three times weekly. Five type I males, three females and three type II males were scanned using CT and varied in size from 105 to 209 mm standard length (SL) (measured from the snout to caudal peduncle). Animals used for micro-computerized tomography (microCT) were housed at the University of Washington

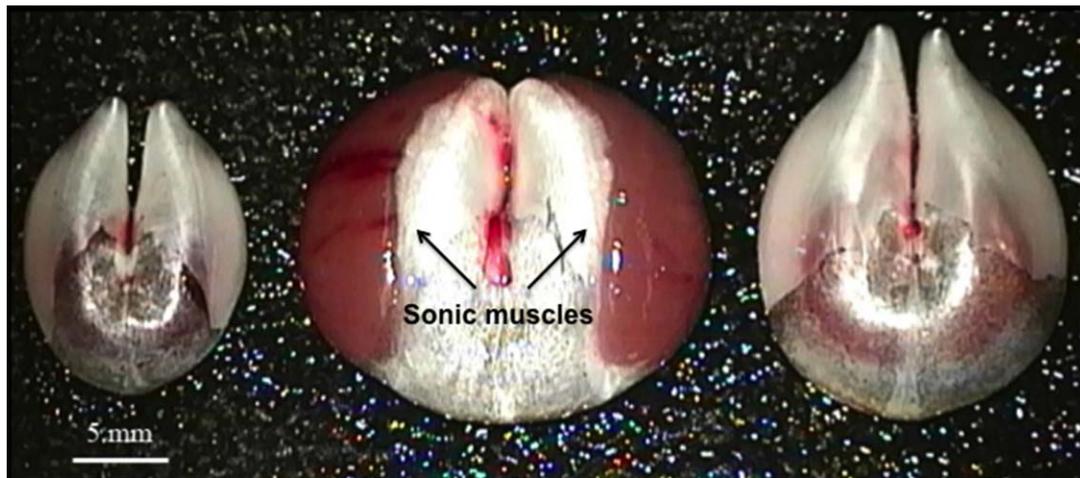


FIGURE 1 Dorsal view of dissected swim bladders from the three sexual phenotypes of the plainfin midshipman (*Porichthys notatus*) of varying standard lengths (SL; from left to right: type II male [SL = 105 mm], type I male [SL = 170 mm], and female [SL = 134 mm]). Note, type I males have enlarged sonic muscles attached to their swim bladders that are used to produce the seasonal advertisement call while females and type II males have greatly reduced sonic muscles in both size and vasculature

where they were maintained at 13°C–14°C on a 14:10 hr light cycle and fed frozen vitamin enriched shrimp three times weekly before being euthanized for microCT-scanning via overdose of ethyl *p*-amino-benzoate in a saltwater bath. Twenty-seven adult type I males, type II males, and females (nine from each sexual phenotype) of similar SL were selected for imaging. While adult plainfin midshipman can vary greatly in size within and across morphs, all animals chosen for microCT-imaging were between 112 and 138 mm to ensure an overlap in size between all three sexual phenotypes.

2.2 | CT-imaging

Initial observations of swim bladder dimorphisms in the plainfin midshipman based on gross dissections (Figure 1) led to a pilot group of animals being imaged using a CT-scanner in the Computerized Scanning and Imaging Facility (<http://csi.whoi.edu>) at the Woods Hole Oceanographic Institution.

Whole animal overviews (topograms) were obtained to assess the positioning for the CT-sequences. Ultrahigh resolution sectional data were obtained on a Siemens Volume Zoom CT unit using a spiral protocol with parameters of 120 kV, 180 mAS, 0.5 mm acquisitions at 0.5 mm table increments. Primary images were formatted in the transaxial plane at 0.1 mm sectioning and a 50 mm field of view (FOV), providing 512 matrix, 100 micron isotropic voxel resolution, in both soft (H40) and ultrahigh bone kernels (H90) with extended scales to enhance soft tissue detail and eliminate image artifacts from hyperattenuating structures such as the otoliths. Some images were also obtained and reconstructed at 1 mm increments at larger FOVs to document the entire body of the fish and assess sexual differences. Composite three-dimensional reconstructions of multiple regions of interest (ROI), such as the gas-filled areas, swim bladder surface, structures, and otoliths, were obtained using Siemens proprietary software, with ROIs segmented by Hounsfield values. The DICOM images and raw data were archived onto magneto-optical disks and additional

DICOM copies were transferred via CDs for off-line use with eFilmLite 3.1 (Merge Healthcare) and OsiriX (MacOS) software.

SL and BM were recorded and the gonads were dissected and weighed to calculate gonadal somatic index (GSI).

2.3 | MicroCT-imaging

Specimens were scanned at the Small Animal Tomographic Analysis Facility at Seattle Children's Research Institute where they were imaged using a Skyscan 1076 microCT scanner at 50kV, 170μA with a scan resolution of 35.26 μm. Whole body X-ray images were used to determine a rostral point just anterior to the inner ear otoliths and a caudal point just posterior to the swim bladder for microCT-imaging. All data were reconstructed using NRecon (v1.6.9.4) with consistent thresholding parameters. Reconstructions were rendered in 3D using the freeware, Drishti v2.6 (Limaye, 2006). Measurements were made in Drishti using the "path" option after landmark placement. For final images, the swim bladders were segmented from the reconstructed data using Drishti Paint v2.5, while otoliths were pseudo-segmented using simple thresholding as their structures represent the highest mineral densities within the fish, with post-rendering clean-up using Drishti. Scalebars were incorporated within Drishti. Images taken in Drishti were then imported into Adobe Photoshop CS5 to optimize contrast and to facilitate appropriate image scaling. After scans were completed, animals were weighed intact before their gonads were dissected and then weighed to calculate GSI and confirm sexual phenotype.

2.4 | Morphometric analysis

Measurements were represent absolute distance in three-dimensional space in millimeters and calculated from the microCT generated data. Measurements were taken from the rostral-most point of the swim bladder to the caudal-most points of the otoliths in each respective

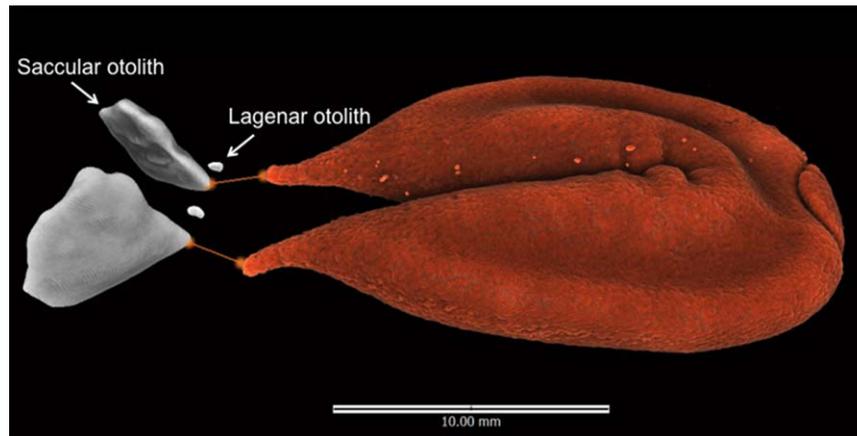


FIGURE 2 Representative image showing the close proximity of the rostral swim bladder extensions to the inner ear otoliths in *Porichthys notatus*. Such images were used to determine the distance between the rostral swim bladder extensions and the otoliths in each of the auditory end organs (only the otoliths of the saccule and lagena are shown in this image while the utricle has been omitted). Note, the lines connecting the swim bladder extensions to the saccular otoliths indicate the calculated distance measurements and are not physical structures

end organ (saccule, utricle, and lagena, see: Figure 2). Swim bladders were also measured for length and width. The rostral swim bladder extensions or “horns” were defined as the anterior projections of the swim bladder that project beyond the round shape of the bladder at the rostral end (Figure 3). This metric was calculated as the difference between the length of swim bladder minus the width or diameter of the round swim bladder, which yields the relative length of the rostral extensions of the swim bladder. All swim bladder to otolith distance measurements were measured bilaterally and averaged within each subject to account for any differences in laterality. In order to account for sexually dimorphic differences in animal size, swim bladder, and the swim bladder extension-to-otolith measurements were divided by the fish’s SL to normalize the measurements, thus creating a normalized distance ratio for all measurements taken. Statistical analyses were conducted using SPSS 19 Software (SPSS, Chicago, IL) and GraphPad Prism 5 (GraphPad Software, La Jolla, CA) with an α set at 0.05. A multivariate analysis of variance (MANOVA) using Wilk’s Lambda multivariate test compared the swim bladder to otolith distance measurements for all three end organs across the three sexual phenotypes. The effect of sexual phenotype on swim bladder size (length and width) and distance between swim bladder extensions and each inner otolith was determined using a one-way analysis of variance (ANOVA) followed by Tukey post hoc tests for planned comparisons.

3 | RESULTS

Initial observations from our gross dissections and preliminary CT-scans of the midshipman swim bladders (Figure 4) revealed remarkable morphological differences in swim bladder morphology among the three sexual phenotypes. Notably, we observed the presence of horn-like projections on the rostral ends of the swim bladders in both females and type II males. In contrast, type I males lacked the prominent horn-like swim bladder extensions and the rostral swim bladder was observed to be more rounded in shape. In addition, the rostral

swim bladder extensions in females and type II males appeared to project closer to the inner-ear end organs (saccule, lagena, and utricle) than in type I males (Figure 4). However, our initial sample sizes of the CT-scanned swim bladders were too few to adequately characterize the morphological differences observed for the three sexual phenotypes. Thus, a more detailed examination of the swim bladders was conducted on a second set of subjects using a microCT-scanner.

3.1 | MicroCT analysis of midshipman swim bladders

MicroCT-analyses of the swim bladders were conducted on 27 plainfin midshipman: nine type I males, nine type II males and nine females. Subjects were selected for microCT analysis based on their SLs in millimeters (mm) to minimize variation between sexual phenotypes. Type I males had a size range of 119–138 mm SL (mean SL = 132 ± 7 mm SD) and 21.4–31.4g BM (mean BM = 28.3 ± 3.1 g SD) and a GSI of 1.7 ± 0.6 SD. Type II males had a size range of 112–121 mm SL (mean SL = 116 ± 3 mm SD) and 15.0–20.5 g BM (mean BM = 17.8 ± 2.1 g SD) and a GSI of 15.6 ± 3.0 SD. Females had a size range of 110–130 mm SL (mean SL = 123 ± 7 mm SD) and 21.7–26.2 g BM (mean BM = 20.9 ± 4.4 g SD) and a GSI of 26 ± 9.0 SD. Type I males were significantly larger (based on SL) than both females ($p < .001$) and type II males ($p < .05$) while females were larger than type II males ($p < .05$; one-way ANOVA, post hoc Tukey test, $F(2, 24) = 16.59$, $p < .001$). Similarly, type I males had a significantly greater BM than both females and type II males ($p < .001$), but females and type II males did not differ in BM ($p = .13$; one-way ANOVA, post hoc Tukey test, $F(2, 24) = 23.75$, $p < .001$).

The swim bladders of the three sexual phenotypes differed in both length and width. Female swim bladders (mean = 22.68 ± 0.95 mm SD) were significantly longer than the swim bladders of type I males (mean = 20.19 ± 1.57 mm SD, $p < .001$) and type II males (mean = 20.85 ± 1.13 mm SD, $p < .05$), while type I and type II males did not differ in swim bladder length ($p = .52$; one-way ANOVA, post hoc Tukey test, F

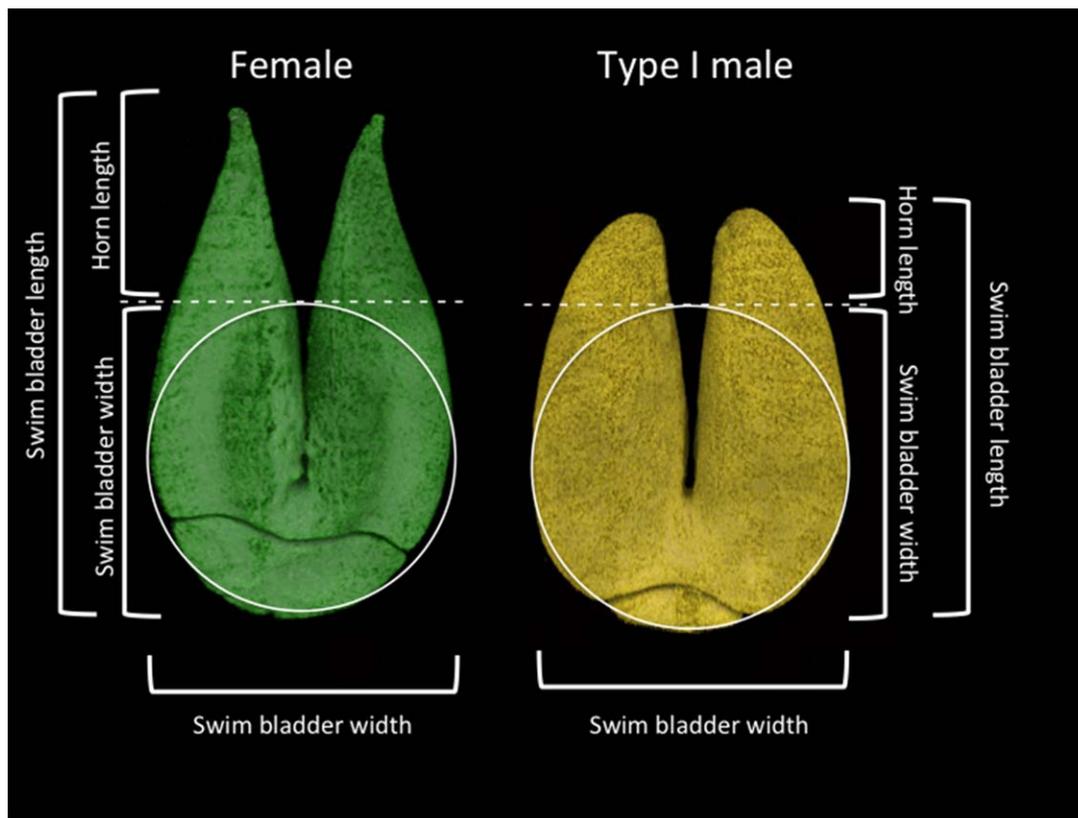


FIGURE 3 Representative images that show the differences in swim bladder morphology between female and type I male in *Porichthys notatus*. Females have horn-like projections on the rostral ends of the swim bladders while type I males lacked such prominent swim bladder extensions. The rostral swim bladder horns were defined as the anterior projections of the swim bladder that projected beyond the primary round shape of the swim bladder and was measured as the difference between the length of the swim bladder minus the width (i.e., diameter of the circle) of the swim bladder. Note that when viewed dorsally, the swim bladder has a primarily round shape as can be seen by the overlaid circles

(2, 23) = 8.82, $p < .001$). When normalized for animal size (SL), the swim bladder length to SL ratios did not differ between females (mean = 0.18) and type II males (mean = 0.18; $p = .52$), however both females and type II males had significantly longer swim bladder to SL ratios than type I males (mean = 0.15; one-way ANOVA, post hoc Tukey test, $F(2, 23) = 33.83$, $p < .001$). Although the swim bladder width of females (mean = 14.10 ± 1.34 mm SD) and type I males (mean = 14.23 ± 0.97 mm SD) did not differ ($p = .96$), the swim bladder widths of females and of type I males were wider than that of type II males (mean = 11.91 ± 0.82 mm SD; one-way ANOVA, post hoc Tukey test, $F(2, 23) = 13.67$, $p < .001$). When normalized for animal size (SL), the swim bladder width to SL ratios neither differ between females (mean = 0.11) and type I males (mean = 0.11; $p = .21$) nor between type II males (mean = 0.10) and type I males ($p = .35$); however, the female swim bladder width to SL ratio was wider than that of type II males (one-way ANOVA, post hoc Tukey test, $F(2, 23) = 4.87$, $p < .05$).

The rostral swim bladder extensions were longer and more prominent in the female and type II male sexual phenotypes (Figure 5). Although the lengths of the rostral swim bladder extensions in females (mean = 8.57 ± 0.51 mm SD) and type II males (mean = 8.93 ± 0.67 mm SD) did not differ ($p = .55$), the swim bladder extensions in both females and type II males were significantly longer than in type I males

(mean = 5.96 ± 0.83 mm SD; one-way ANOVA, post hoc Tukey test, $F(2, 23) = 49.69$, $p < .001$, see Figure 5). When normalized for animal size (SL), the rostral swim bladder extension length to SL ratio in type II males (mean = 0.77) was longer than that of females (mean = 0.70; $p < .05$) and type I males (mean = 0.45; $p < .001$) while the swim bladder extension length to SL ratio in females was also longer than that of type I males (one-way ANOVA, post hoc Tukey test, $F(2, 23) = 81.08$, $p < .001$).

The distance between the rostral swim bladder extensions and the otoliths in the three inner ear end organs (sacculle, lagena, and utricle) differed across sexual phenotype (MANOVA, effect of sexual phenotype, $F(6,44) = 11.41$, $p < .001$; Figure 6). Although there was no difference in the distance between the swim bladder extensions and the saccular otoliths between females (mean = 2.59 ± 0.52 mm SD) and type II males (mean = 2.02 ± 0.25 mm SD; $p = .27$), the distance between the swim bladders extensions and the otoliths in the sacculle, which is the primary auditory end organ in the midshipman, was significantly shorter (approximately $\frac{1}{2}$ the distance) in females and type II males compared to type I males (mean = 5.21 ± 1.20 mm SD; one-way ANOVA, post hoc Tukey test, $F(2, 24) = 43.78$, $p < .001$; Figure 6b). When the swim bladder extension to saccular otolith distance was normalized based on SL, females (mean = 0.021) and type II males (mean = 0.017) had significantly shorter distances between swim bladder

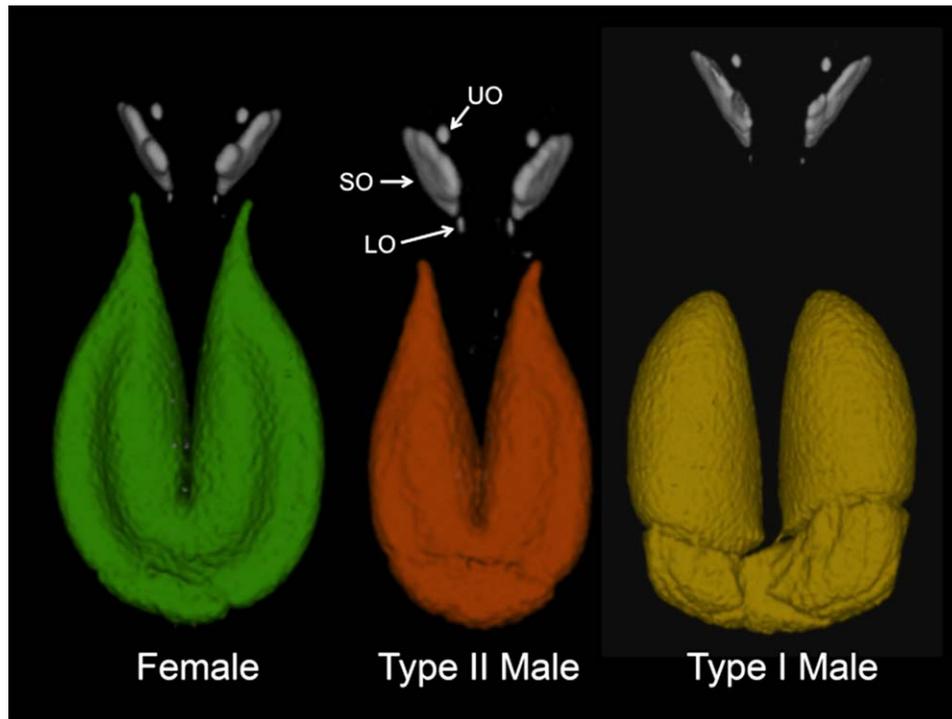


FIGURE 4 Representative computerized tomography (CT) scans of a female, type I and II male that show differences in swim bladder shape and relative proximity of the swim bladder to the inner ear otoliths of *Porichthys notatus*. The largest otoliths reside in saccule (SO), the main end organ of hearing in this species, while ventro-caudal to the saccular otoliths are the otoliths of lagena (LO), also a putative midshipman auditory end organ, and then medial-anterior to the saccular otoliths are the otoliths of the utricle (UO), a putative vestibular and auditory end organ. These swim bladder CT scans are from animals that varied in size (i.e., standard length [SL]) from 105 to 209 mm (female SL = 134 mm, type II SL = 105 mm, and type I SL = 209 mm)

extensions to saccular otoliths than type I males (mean = 0.040) with no difference ($p = .20$) between females and type II males (one-way ANOVA, post hoc Tukey Test, $F(2, 24) = 39.52, p < .001$).

The distance between the rostral swim bladder extensions and the otoliths of the lagenae was significantly shorter in females (mean = 2.89 ± 0.16 mm SD) and type II males (mean = 2.29 ± 0.22 mm SD) compared to type I males (mean = 4.71 ± 1.05 mm SD) with no difference ($p = .16$) observed between females and type II males (one-way ANOVA, post hoc Tukey Test, $F(2, 24) = 30.79, p < .001$; Figure 6c). When normalized for SL, females (mean = 0.024) and type II males (mean = 0.020) had significantly shorter distances between swim bladder extensions to lagenar otoliths than type I males (mean = 0.036) with no difference ($p = .14$) between females and type II males (one-way ANOVA, post hoc Tukey Test, $F(2, 24) = 23.65, p < .001$).

The distance between the rostral swim bladder extensions and the otoliths of the utricles was also significantly shorter in females (mean = 5.24 ± 0.76 mm SD) and type II males (mean = 5.01 ± 0.50 mm SD) than in type I males (mean = 8.84 ± 1.22 mm SD) with no difference ($p = .84$) observed between females and type II males (one-way ANOVA, post hoc Tukey Test, $F(2, 24) = 53.55, p < .001$; Figure 6d). When normalized for SL, females (mean = 0.042) and type II males (mean = 0.043) had significantly shorter distances between swim bladder extensions to utricle otoliths than type I males with no difference ($p = .76$) between females and type II males (mean = 0.067; one-way ANOVA, post hoc Tukey Test, $F(2, 24) = 45.76, p < .001$).

4 | DISCUSSION

The goal of this study was to characterize the morphology of the swim bladder and quantify the relative distance between the swim bladder and inner ear organs in the three sexual phenotypes (females, type I and type II males) of the plainfin midshipman fish, *P. notatus*. Our results show that females and type II males have prominent horn-like extensions on the rostral ends of the swim bladders that extend toward the auditory end organs. These rostral swim bladder extensions were longer in both females and type II males compared to that of type I males. In addition, the distance between the rostral swim bladder extensions and each inner-ear end organ (saccule, lagena, and utricle) was significantly shorter in females and type II males compared to that in type I males, and this decreased distance between the swim bladder and auditory end organs likely increases auditory sensitivity to sound pressure and extends the upper range of frequencies that can be detected.

4.1 | Functional significance of the swim bladder in female and type II male midshipman

Previous studies have reported sexual dimorphisms in the swim bladders of South Asian torrent minnows (Psilorhynchidae; Conway, Britz, & Siegel, 2014), Atlantic croakers (Sciaenidae; Hill, Fine, & Musick, 1987), oyster toadfish (Batrachoididae; Fine et al., 1990) along with

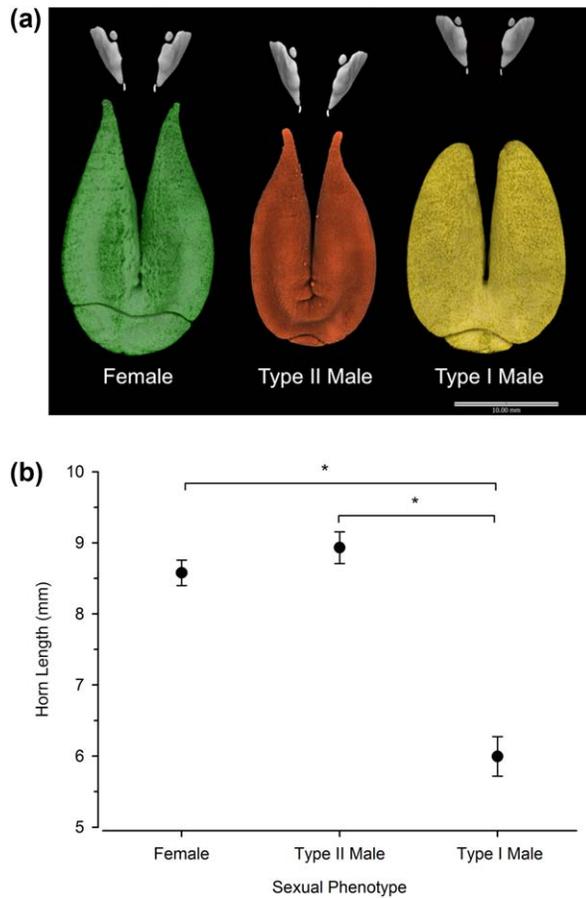


FIGURE 5 Micro computerized tomography (microCT) scans and analysis of the swim bladder horn extensions in the sexual phenotypes (females, type I and II males) of *Porichthys notatus*. (a) Representative microCT scans showing the dorsal view of the swim bladders along with the inner ear otoliths in midshipman females, type I and II males. All three of the scanned sexual phenotypes were similar in size (standard length measurements: female = 119 mm, type II male = 118 mm, and type I male = 125 mm). (b) Comparison of swim bladder horn length across the three midshipman sexual phenotypes. All data are plotted as mean \pm SE, * $p < .001$

several species of cusk eels (Ophidiidae) with implications for sound production (Courtenay, 1971; Kéver et al., 2012; Rose, 1961). While Bass and Marchaterre (1989) reported overall size differences in gross dissections of the swim bladders of plainfin midshipman across sexual phenotypes, no study has yet provided evidence for functional differences in swim bladders between the sexes, nor between alternative sexual phenotypes of a given sex as it relates to sound reception. Our results of intrasexual and intersexual dimorphic differences in swim bladder morphology suggest that these differences may be functionally related to differences in sound pressure sensitivity, especially as it relates to social behavior in this species. We show that females and type II males have rostral swim bladder extensions that were 1.4 to 1.5 times longer than those in type I males and the presence of these swim bladder extensions results in a shorter distance between the swim bladder and inner ear otoliths (especially the sagitta of the sacculus and the asteriscus of the lagena).

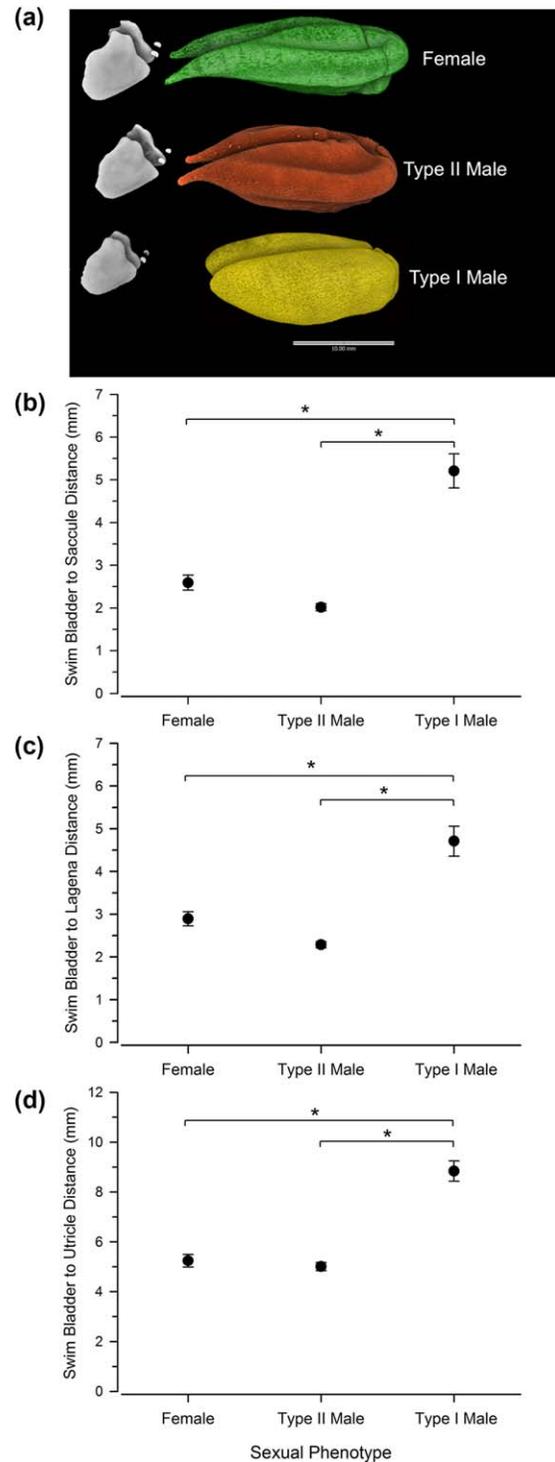


FIGURE 6 Micro computerized tomography (microCT) scans and analysis of the swim bladder horn extensions and their proximity to the otoliths of the inner end organs (sacculus, lagena, and utricle) in *Porichthys notatus*. (a) Representative microCT scans showing a lateral view of the swim bladder and inner ear otoliths. Note that only the otoliths of the sacculus and lagena are visible in this lateral view. (b–d) Comparisons of the distance between the rostral points of the swim bladder and the caudal points of the (b) saccular otoliths, (c) lagenar otoliths, and (d) utricular otoliths. Measurements are taken bilaterally and averaged within each animal. All data are plotted as mean \pm SE, * $p < .001$

The presence of rostral horn-like swim bladder extensions has been previously observed in other fishes from several teleost families including Holocentridae, Gadidae, Gerreidae, Sciaenidae Chaetodontidae, and more recently Cichlidae (Braun & Grande, 2008; Hawkins, 1986; Ladich, 2008; Nelson, 1955; Parmentier, Mann, & Mann, 2011; Ramcharitar, Higgs, & Popper, 2006; Tricas & Webb, 2016). These previously reported swim bladder extensions have been posited to enhance sound pressure sensitivity by providing closer proximity of the swim bladder to the auditory end organs (Braun & Grande, 2008; Fine & Parmentier, 2015; Parmentier et al., 2011; Ramcharitar et al., 2006; Schulz-Mirbach, Martin, Metscher, & Ladich, 2012; Schulz-Mirbach, Metscher, & Ladich, 2012). While some fishes such as otophysan fishes (e.g., goldfish [*Carassius auratus*] and zebrafish [*Danio rerio*]) have specialized skeletal adaptations known as Weberian ossicles that directly link the swim bladder to the inner ear for sound pressure sensitivity, other fishes such as holocentrids and gadids (i.e., squirrelfishes and cods, respectively) possess paired rostral swim bladder extensions that approach the skull and provide increasing pressure sensitivity the closer the swim bladder extensions are to the inner-ear end organs (Chapman & Hawkins, 1973; Coombs & Popper, 1979). The close proximity of the swim bladder to the inner ear afforded by the rostral swim bladder extensions is thought to allow the auditory end organs (e.g., saccule and lagena) to detect the local particle motion produced by pressure-wave induced vibrations of the swim bladder when exposed to sound. This indirect mechanism for sound pressure detection is posited to increase overall auditory sensitivity and extend the upper range of acoustic frequencies that are detected by the fish (Sisneros & Rogers, 2016; Tricas & Webb, 2016).

The distance between the horn-like swim bladder extensions and the otoliths of the saccule and lagena in the midshipman was less than 3 mm in both females and type II males compared to a distance greater than 4.7 mm for the same measurements in type I males. Similar studies of sciaenids (Ramcharitar et al., 2006), ophidiids (Kéver et al., 2014), and cichlids (Schulz-Mirbach et al., 2012), species with swim bladders less than 3 mm away from the otic capsule, which contains the inner ear end organs, showed enhanced auditory sensitivity to acoustic stimuli over 1500 Hz. In addition, Schulz-Mirbach et al. (2012) showed that in cichlids the size of the swim bladder may also affect hearing such that fish with larger swim bladders could detect higher frequencies while the presence of rostral swim bladder extensions increased overall auditory sensitivity to acoustic stimuli between 500 and 1000 Hz. In our study, we show that the absolute swim bladder length was longer in females than in type I and type II males, however when normalized for standard body length (SL), both females and type II males had significantly larger swim bladder to SL ratios than type I males. Taken together, our findings predict that the relatively long rostral horn-like swim bladder extensions and larger swim bladders in midshipman females and type II males should facilitate enhanced sound pressure sensitivity and increase the upper range of detectable frequencies in these two sexual phenotypes. Future studies that investigate saccular and lagenar auditory physiology of females and type II males that involve experimental deflation or removal of the swim bladder will be

informative as to the role of the swim bladder in sound pressure sensitivity in this species.

The predicted enhancement of sound pressure sensitivity and higher frequency detection in females and type II male midshipman would likely increase the detection of social acoustic signals in shallow water environments like those in which midshipman breed and interact during the reproductive season. Plainfin midshipman fish are known to produce three types of vocalizations that include hums, growls, and grunts. These vocalizations are produced during social and reproductive behaviors and are generated by the contraction of the sonic muscles attached to the swim bladder. During the breeding season, type I males produce the multiharmonic hum or advertisement call to attract females for spawning. The hum contains a fundamental frequency that ranges from 80 to 102 Hz with harmonics that can extend up to 1000 Hz (Bass, Bodnar, & Marchaterre, 2003; Brantley & Bass, 1994; Ibara, Penny, Ebeling, van Dykhuizen, & Cailliet, 1983). Growls and grunt trains, which are agonistic calls also produced by type I males, are broadband signals that contain frequency information up to 800 Hz (Bass et al., 1999; Maruska & Sisneros, 2015). Previous studies have shown that females and type I and II males exhibit an adaptive form of reproductive-state dependent auditory plasticity that results in increased sensitivity during the reproductive season to a broad range of frequencies including the dominant higher frequencies in type I male vocalizations (Bhandiwad, Whitchurch, Colleye, Zeddies, & Sisneros, 2017; Rohmann & Bass, 2011; Sisneros, 2009; Sisneros & Bass, 2003). In shallow waters (<3- to 4-m deep), the relatively high frequencies (>180 Hz) contained within midshipman vocalizations (hum, grunts, and growls) will propagate farther than the calls' fundamental frequency due to the inverse relationship between water depth and the cutoff frequency of sound transmission (Bass & Clark, 1989; Fine & Lenhardt, 1983; Rogers & Cox, 1988). The mechanism responsible for the seasonal plasticity of auditory sensitivity in females and type I males is known to be due in part to seasonal fluctuations in circulating steroid hormones; that is, testosterone and 17 β -estradiol (Forlano, Sisneros, Rohmann, & Bass, 2015; Rohmann & Bass, 2011; Sisneros et al., 2004). In addition, females are known also to exhibit seasonal changes in saccular hair cell density that are concurrent with changes in hair cell sensitivity, which may be another contributing factor to the seasonal increase in auditory sensitivity (Coffin et al., 2012). Another morphological feature that may enhance the auditory sensitivity of the saccule is the mass of the saccular otolith. A recent study of midshipman saccular otoliths revealed that females and type II males have sagittae (saccular otoliths) that are larger and of greater mass than those in type I males (Bose, Adragna, & Balshine, 2016). Otoliths with greater mass are thought to improve auditory sensitivity especially at low frequencies; as otolith mass becomes heavier, auditory sensitivity increases and the frequency at which otolith amplitude displacement is greatest shifts toward lower frequencies (Lychakov & Rebane, 2000). Thus, the sagittae with greater mass in females and type II males may be another potential structure that affects sound pressure detection, given heavier mass loaded otoliths in females and type II males may be more sensitive to the local particle motion produced by the pressure

induced oscillations of the swim bladder during sound exposure. Finally, the increased pressure sensitivity afforded by the horn-like swim bladder extensions in females and type II males should also aid in the localization of sound sources; that is, vocalizing conspecifics. Recent behavioral experiments that investigated the role of pressure reception by females using the swim bladder during sound source localization revealed that sound pressure reception is likely required for the localization of sound sources (based on the results of females performing phonotaxis to a sound source with intact versus deflated swim bladders; Coffin et al., 2014). In summary, the predicted enhanced sound pressure sensitivity in females and type II males due to the rostral swim bladder extensions may be yet another potential mechanism that enhances the probability of detection and localization of conspecifics during social behaviors in the reproductive season.

4.2 | Functional significance of the swim bladder in type I males

As with the other sexual phenotypes, type I males produce sound by contracting their sonic muscles that are attached to the swim bladder. Although females and type II males are capable of producing grunts, only type I nesting males can produce sustained grunt trains and long duration growls and hums. The hum or advertisement call can be produced by type I males for over an hour in duration and at relatively high sound levels (Bass et al., 1999). The sound levels of type I male hums have been recorded to be as high as 153–161 dB (re 1 μ Pa) at or near the entrance of the nests from captive calling males maintained in artificial nests at the Friday Harbor Laboratories on San Juan Island, WA (personal observations, JAS). Overexposure to loud sounds for relative long periods of time can have potentially damaging effects on the auditory system and lead to temporary and/or permanent deficits in auditory sensitivity and perception (Le Prell, Henderson, Fay, & Popper, 2012). The swim bladder shape and distance from the sacculle and lagena in type I males may be adaptive in terms of reducing sound overexposure of the inner ear in nesting type I males during the breeding season as has been suggested for males of the closely related oyster toadfish (*Opsanus tau*) which lack swim bladder extensions (Barimo & Fine, 1998). Furthermore, experimental deflations of male swim bladders in *O. tau* have revealed no differences in hearing thresholds versus animals with intact swim bladders (Yan, Fine, Horn, & Colon, 2000) supporting the idea that swim bladders are adapted primarily for sound production in males. The distance between the swim bladder and otoliths of the sacculle and lagena in type I males was approximately 1.5 times greater than that in females and type II males. In addition, the normalized swim bladder length of type I males was approximately 17% shorter than that of females and type II males while the normalized swim bladder width of type I males did not differ from that of females and type II males. Thus, the increased distance between the swim bladder and the saccular and lagenar end organs coupled with a smaller normalized swim bladder may act to reduce sound pressure sensitivity and higher frequency stimulation of the inner ear in type I males from sounds that are both self-generated and produced from nearby calling males. While it is possible that type I males have

swim bladders of similar shape to females and type II males early in development, future studies that investigate how ontogeny affects swim bladder shape and proximate distance to the inner ear end organs in type I males will be needed to determine if there are life history dependent changes in sound pressure sensitivity in this male sexual phenotype.

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