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Ontogeny of Inner Ear Saccular Development in the Plainfin Midshipman (*Porichthys notatus*)

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Abstract
The auditory system of the plainfin midshipman fish (*Porichthys notatus*) is an important sensory system used to detect and encode biologically relevant acoustic stimuli important for survival and reproduction including social acoustic signals used for intraspecific communication. Previous work showed that hair cell (HC) density in the midshipman saccule increased seasonally with reproductive state and was concurrent with enhanced auditory saccular sensitivity in both females and type I males. Although reproductive state-dependent changes in HC density have been well characterized in the adult midshipman saccule, less is known about how the saccule changes during ontogeny. Here, we examined the ontogenetic development of the saccule in four relative sizes of midshipman (larvae, small juveniles, large juveniles, and nonreproductive adults) to determine whether the density, total number, and orientation patterns of saccular HCs change during ontogeny. In addition, we also examined whether the total number of HCs in the saccule differ from that of the utricle and lagena in nonreproductive adults. We found that HC density varied across developmental stage. The ontogenetic reduction in HC density was concurrent with an ontogenetic increase in macula area. The orientation pattern of saccular HCs was similar to the standard pattern previously described in other teleost fishes, and this pattern of HC orientation was retained during ontogeny. Lastly, the estimated number of saccular HCs increased with developmental stage from the smallest larvae (2,336 HCs) to the largest nonreproductive adult (145,717 HCs), and in nonreproductive adults estimated HC numbers were highest in the saccule (mean ± SD = 28,479 ± 4,809 HCs), intermediate in the utricle (mean ± SD = 11,008 ± 1,619 HCs) and lowest in the lagena (mean ± SD = 4,560 ± 769 HCs).

Introduction

The teleost inner ear is composed of three semicircular canals and three putative auditory end organs that include the saccule, lagena, and utricle. Each auditory end organ contains a single, dense calcium carbonate otolith that rests on a sensory bed of hair cells (HCs; sensory macula) that responds to linear acceleration and functions as an inertial accelerometer [for reviews, see Popper and Fay, 1993; Popper and Lu, 2000]. These otolithic end organs continue to grow in fish throughout early development and into the adult life history stage. As the inner ear sensory epithelium grows, HCs are continuously added, and the total number of HCs in the macula increases.

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This increase in total HCs generally coincides with a reduction in HC density because the rate of HC addition is often less than the rate of macular epithelial growth [Popper and Hoxter, 1984; Lombarte and Popper, 1994, 2004; Chaves et al., 2017; but see Lu and DeSmidt, 2013, and Wang et al., 2015]. In addition, as HCs are continuously added to sensory epithelia in the auditory end organs, ontogenetic changes in the size and shape of the macula may also occur [Corwin, 1983; Popper and Hoxter, 1984; Lombarte and Popper, 1994]. These changes in the size and shape of the macula coupled with the continuous addition of HCs likely contribute to ontogenetic changes in auditory sensitivity of the fish saccule.

More recently, a novel form of saccular-specific HC addition was reported to occur seasonally in the plainfin midshipman fish (*Porichthys notatus*) [Coffin et al., 2012; Lozier and Sisneros, 2019], which is a vocal species of marine teleost fish that has become a good model species to investigate mechanisms of acoustic communication and sound localization [Bass and McKibben, 2003; Sisneros, 2009; Sisneros and Rogers, 2016]. This novel, seasonal increase in HC addition in the saccule, measured as an increase in HC density, was shown to occur in both the reproductive female and nest guarding (type I) male plainfin midshipman [Coffin et al., 2012; Lozier and Sisneros, 2019]. Furthermore, the seasonal increase in HC density in reproductive females was shown to be concurrent with an increase in saccular HC auditory sensitivity [Coffin et al., 2012]. Although seasonal changes in HC density have been well documented in the adult midshipman saccule, less is known about ontogenetic changes in the midshipman saccule.

The focus of this study was to investigate the ontogenetic morphological changes in the saccule of the plainfin midshipman as they may relate to ontogenetic, auditory physiological changes described in prior studies [Sisneros and Bass, 2005; Alderks and Sisneros, 2011]. Previous work with the plainfin midshipman fish, which is a close relative of the toadfish and belongs to the same family Batrachoididae, has yielded conflicting results in terms of how peripheral auditory sensitivity changes during ontogeny. Alderks and Sisneros [2011] showed based on saccular potential recordings that midshipman fish exhibit no change in auditory sensitivity during ontogeny but instead show an ontogenetic retention of auditory sensitivity from small juveniles to adults. In contrast, Sisneros and Bass [2005] showed based on saccular afferent recordings that auditory sensitivity increased with size/age from small juveniles to adults. These results suggest that the midshipman does exhibit ontogenetic increases in auditory sensitivity at least at the level of auditory afferents postsynaptic to the HCs. Whether these results are also related to developmental changes in the saccule remains to be determined.

The goal of this study was to characterize the ontogenetic development of the saccule in the plainfin midshipman (*P. notatus*). We examined how the density, total number, and orientation patterns of HCs in the saccule change during ontogeny from larvae to adults. In addition, we also assessed how the saccular epithelia change in size and shape during ontogenetic development. A secondary aim was to examine the total number of HCs in the utricle and lagena of adults to compare with that of the adult saccule. We discuss the potential functional significance of our findings and how they may relate to ontogenetic changes in the sensitivity of the auditory system in the plainfin midshipman.

Methods

Animal Collection and Care

All large juvenile (n = 11) and nonreproductive adult (n = 30) midshipman fish were collected via otter trawl in Monterey Bay near Moss Landing, CA, during the month of January in 2018 and 2020. Large juveniles were distinguished from adults based on standard length (SL), which were less than 10.5 cm for males and less than 8.5 cm for females but larger than 3 cm for both males and females. Small juveniles (n = 20) (<3 cm SL) were raised from larvae incubated in captivity. Midshipman larvae were collected from Hood Canal near Brinnon, WA, and Tomales Bay near Marshall, CA, in May-July 2019. Larvae were defined as any fish still nourishing on yolk (n = 11). Some larvae were held in tanks until all yolk was absorbed and they became free-swimming juveniles, up to 3 months after collection. The size ranges used in this study were similar to those used in previous midshipman studies [Brantley et al., 1993; Bass et al., 1996; Grober et al., 1994; Foran and Bass, 1998; Sisneros, 2007; Alderks and Sisneros, 2011]. All midshipman fish were maintained in 50-gallon recirculating saltwater tanks at approximately 25 parts per thousand salinity and an average temperature of 13.5 °C. Winter-collected adult and large juvenile midshipman fish were held in tanks for 4.5 weeks or less after capture. Adults/large juveniles were maintained with an 8-h light/16-h dark photoperiod, and larvae/small juveniles were maintained with a 12-h light/12-h dark cycle to simulate the photoperiod during the time of year in which the animals were collected. Adults and free-swimming juveniles were fed deshelled raw shrimp 2–3 times per week.

Tissue Collection

Prior to tissue collection all large juvenile and adult animals were anesthetized in a 10% benzocaine saltwater bath, transcardially perfused with teleost ringer solution followed by 4% paraformaldehyde dissolved in 0.1 M phosphate buffer (PB) solution, and then all auditory end organs (saccule, lagena, and utricle) were removed from the otic capsule via a dorsal craniotomy. Auditory end organs were then immersed and postfixed with 4% paraformaldehyde dissolved in 0.1 M PB for 1 h. Larvae and small juveniles were not perfused but were sacrificed via benzocaine overdose. Auditory end organs were then immediately removed from the otic capsule following a dorsal craniotomy and immersion fixed

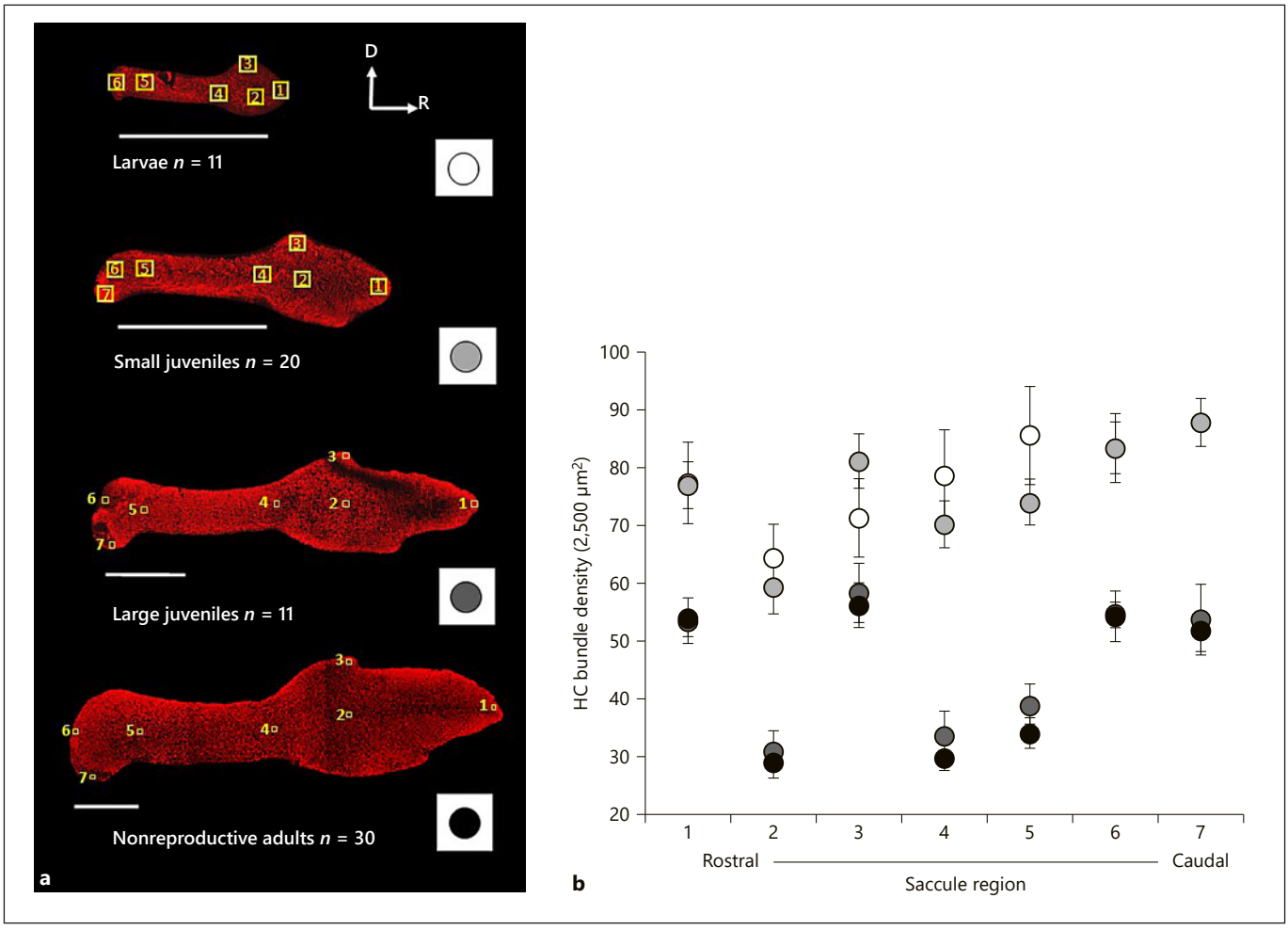

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Fig. 1. Saccular HC density by region in larvae, small juveniles, large juveniles, and nonreproductive adult midshipman fish. **a** Representative saccular maculae from larval, small juvenile, large juvenile, and nonreproductive adult plainfin midshipman. HC bundles were counted in 6 discrete 50 × 50 µm regions in larvae and 7 discrete regions of the same dimension in small juveniles, large juveniles, and nonreproductive adults. Counts were not made in region 7 in larvae because this region had not yet developed in the saccular macula. All boxes are drawn to scale. D, dorsal; R, rostral; white scale bars, 500 µm. **b** Average HC bundle density in each region for each developmental stage. Each number on the *x* axis corresponds to the number of each region shown in **a**. White circles, larvae; light gray, small juveniles; dark gray, large juveniles; black, nonreproductive adults. For statistical differences see Results section. Error bars represent ±95% confidence intervals.

with 4% paraformaldehyde dissolved in 0.1 M PB for 2 h. Saccules, lagenae, and utricles of all developmental stages were washed in 0.1 M PB following immersion fixation. Sensory epithelia were isolated by removing the otolith and otolith membrane using forceps. End organ epithelia were kept in 0.1 M PB with 0.03% sodium azide at 4 °C for 2 months or less prior to staining and imaging for the majority of tissue, with the exception of 3 fish collected in January 2018 for which tissue was stored in the same conditions for 26 months. Following end organ removal, all fish were weighed and measured for SL, and then the gonads were dissected and weighed. Adult fish were sexed based on the presence of ovaries or testes. Juvenile fish were sexed based on the presence (female) or absence (male) of oocytes in the gonadal tissue. There was no way to distinguish between type I and type II males in juveniles as the weights of testes in this developmental stage were undetectable. Visual observation of oocytes could not be detected in larvae, and therefore sex could not be determined at this stage of development.

End Organ Staining

The sensory epithelium of each end organ was washed in a 0.1 M phosphate-buffered saline (PBS) solution and then stained with phalloidin (Invitrogen cat. No. R415) at a dilution of 1:40 in PBS for 1 h. Epithelia were washed again in 0.1 M PBS, placed on coverslips with the apical side against the glass and mounted to slides in Fluoromount-G mounting media (Southern Biotech cat. No. 0100-01). Slides were sealed with nail polish and were stored away from light at 4 °C until imaging.

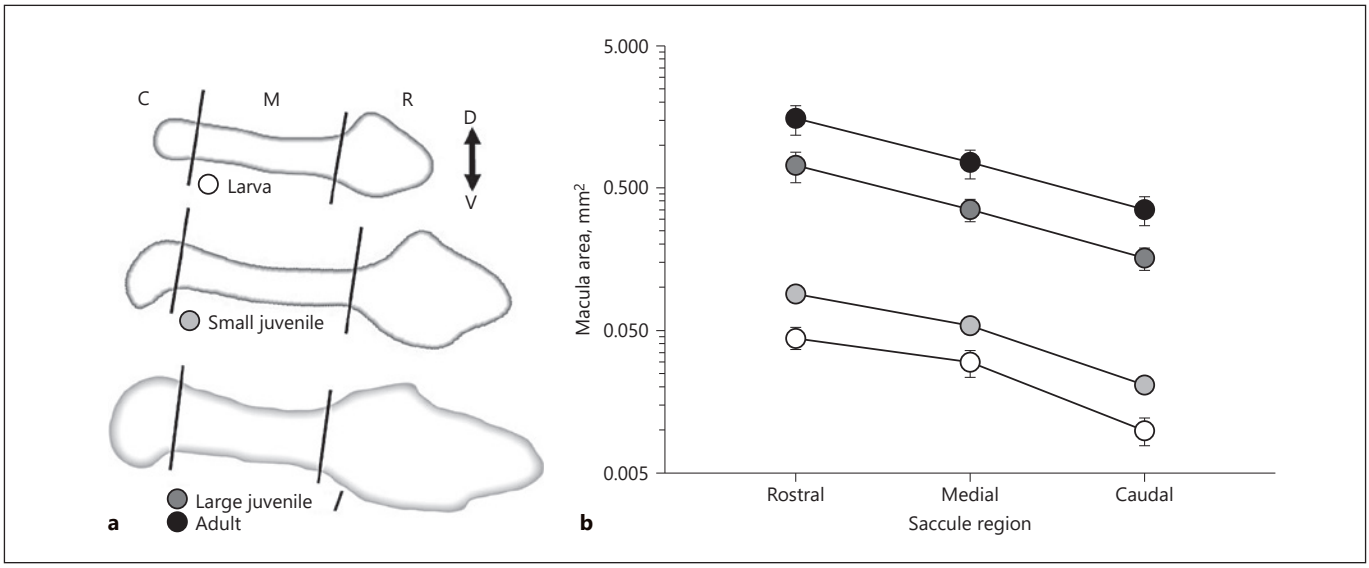

Fig. 2. Comparisons of saccule area and shape. **a** To determine differences in saccular shape, the macula was separated into caudal (C), medial (M), and rostral (R) areas. Sketches are not drawn to scale. V, ventral; D, dorsal. **b** Log-transformed saccule areas were significantly different between all developmental stages with adults having the largest area and larvae having the smallest area. Error bars represent ±95% confidence intervals.

Fluorescence Imaging

All epithelia were imaged on a Leica SP5 confocal microscope with an inverted stand (Leica Microsystems, Buffalo Grove, IL, USA). The phalloidin used for staining was conjugated to rhodamine, so the laser was set to 560/570–620 excitation/emission spectral filter. Images used for HC counts and HC orientation were at 40 × 1.25 NA oil immersion objective for all developmental stages. Images analyzed for area measurements were performed using the 10 × 0.4 NA dry objective for adults and large juveniles, and the 20 × 0.7 NA dry objective for small juveniles and larvae. All images were captured in z-stacks at 0.5-µm increments using Leica Application Suite Advanced Fluorescence software (Leica).

HC Counts/HC Orientation

End organ HCs were counted using Image-J software. Saccule HC counts were collected from 50 × 50 µm squares in 7 discrete regions for nonreproductive adults and juveniles. The 7 discrete regions chosen were similar to those used previously in adult female and type I male midshipman fish [Lozier and Sisneros, 2019], and they represent epithelial regions in both the marginal zone of the macula (peripheral regions 1, 3, 6, and 7) and the central zone of the macula (inner regions 2, 4, and 5). Both marginal and central zones of end organ maculae have been described in previous studies in other species [Corwin, 1981; Popper and Hoxter, 1984] and in adult midshipman fish [Coffin et al., 2012; Lozier and Sisneros, 2019]. In addition, HC density was also examined in the rostral zone (anterior regions 1, 2, 3, and 4) and caudal zone (posterior regions 5, 6, and 7) of the saccular macula. In larvae, HC counts were collected from only six 50 × 50 µm regions because epithelial region 7 was not present in the saccule of larvae (Fig. 1). Six of the saccular epithelia from fish collected in January 2018 were stained and used for counts in a previous study [Lozier and Sisneros, 2019]. We used these same images for this study, but HCs were recounted in this experiment. HC counts were collected from three 50 × 50 µm squares in utricles and four 50 × 50 µm squares in lagenae. In our analysis, HCs were defined as separate stereocilia bundles. Bundles that were only partially within the counting box were included in the analysis. HC orientation in saccules was determined by imaging each HC at the level of the cuticular plate. The kinocilium does not contain f-actin and therefore is not stained by phalloidin. The kinocilium appears as a black circle on the cuticular plate, and the planes of maximum polarity of the HC stereocilia were determined visually (Fig. 3a).

Statistical Analyses

To compare HC densities between each developmental group, we ran a two-way mixed ANOVA with developmental stage (4 levels: larvae, small juveniles, large juveniles, and adults) as the between-subjects factor and saccule region (6 levels for each region, excluding region 7 because this region is not present in larvae) as the within-subjects factor. We then conducted simple main effects (one-way ANOVAs) followed by Bonferroni corrected pairwise comparisons to determine the effect of developmental stage on HC density at each saccule region.

Differences in saccular macula shape throughout ontogeny were examined by measuring the area of rostral, medial, and caudal maculae (Fig. 2a). Areas were log-transformed to meet the assumption of equality of variances and were compared in a two-way mixed ANOVA. Sizes (area) of each zone were compared between developmental groups with Bonferroni post hoc tests.

To determine whether there was a significant difference in estimated HC quantities between the three end organs (saccule, utricle, and lagena) we conducted a one-way ANOVA with Bonferroni post hoc tests in size-matched adult midshipman fish. Estimated HC numbers were log-transformed to meet the assumption of equality of variances.

Table 1. Mean HC density/region for each developmental stage

Developmental stage	Saccule region						
	1	2	3	4	5	6	7
Larvae	78.2±14.8	64.2±12.4	68.2±8.7	81.2±10.9	83.6±16.2	83.2±10.0	–
Small juveniles	76.1±8.3	57.8±10.1	78.9±9.3	67.1±8.3	73.7±8.1	81.7±6.1	87.8±8.0
Large juveniles	53.5±6.7	30.9±6.0	58.3±8.7	33.6±7.3	38.8±6.2	54.3±7.4	53.7±9.7
Adults	54.0±9.2	29.1±7.6	56.9±9.8	29.7±5.9	33.4±7.3	54.5±6.2	51.7±9.3

Values show mean HC density/region ± SD.

Estimated Hair Cell Numbers

Saccule, lagena, and utricle macula areas were measured using the tracing tool in Image-J. HC numbers were estimated in all three end organs using the following equation described in Popper and Hoxter [1984]:

Total estimated HCs = $(\Sigma HC_C)(A_C/\Sigma A_C$ macula regions) + $(\Sigma HC_m)(A_m/\Sigma A_m$ macula regions),

where $\Sigma HC_C/HC_m$ = total counted HC bundles in central/marginal macula, A_C/Am = area of the central/marginal macula (in μm^2), and $\Sigma A_C/A_m$ macula regions = the summed area of all 50 × 50 μm boxes in each zone of the macula.

Note that for the utricle and lagena, central macula = nonstriolar and marginal macula = striolar. Like marginal regions in the saccule, the striolar regions in these end organs contain greater bundle density than the nonstriolar area [Coffin et al., 2012].

Because statistical evidence (not reported) indicated that HC bundle density differed between caudal and rostral zones in saccules of larvae and small juveniles, and because we could not reliably delineate between the marginal and central zones visually in Image-J, estimated saccule HC numbers in these developmental stages were calculated using the following equation [Lombarte and Popper, 1994; Chaves et al., 2017]:

Total estimated HCs = $(\Sigma HC_r)(A_r/\Sigma A_r$ macula regions) + $(\Sigma HC_c)(A_c/\Sigma A_c$ macula regions),

where $\Sigma HC_r/HC_c$ = total counted HC bundles in rostral/caudal macula, A_r/A_c = area of the rostral/caudal macula (in μm^2), and $\Sigma A_r/A_c$ macula regions = the summed area of all 50 × 50 μm boxes in each zone of the macula.

Note that because no counting regions were located within the medial zone (Fig. 1), the area of the medial zone was evenly divided and added to the rostral and caudal zones for these estimates.

Results

Morphometrics

We sampled a total of 72 plainfin midshipman fish: 11 larvae, 20 small juveniles, 11 large juveniles, and 30 nonreproductive adults. The larvae (sex could not be determined; n = 11) had a mean SL of 1.7 ± 0.2 cm SD (range = 1.5–2.1 cm) and body mass (BM) of 0.13 ± 0.01 g SD. For small juveniles, males (n = 12) had a mean SL of 2.5 ± 0.2 cm SD (range = 2.3 to 2.8 cm) and a mean BM of 0.18 ± 0.04 g SD while females (n = 8) had a mean SL of 2.6 ± 0.2 cm SD (range = 2.2–2.9 cm) and a mean BM of 0.17 ± 0.04 g SD. For large juveniles, males (n = 9) had a mean SL of 8.4 ± 1.3 cm SD (range = 6.6–10.4 cm) and a mean BM of 5.65 ± 3.08 g SD while females (n = 2) had a mean SL of 7.0 ± 0.2 cm SL (range = 6.8–7.1 cm), a mean BM of 2.91 ± 0.16 g SD, and a mean gonadosomatic index (GSI) of 3.1 ± 3.4 SD. For nonreproductive adults, type I males (n = 15) had a mean SL of 14.9 ± 5.8 cm SD (range = 10.6–30.5 cm), a mean BM of 52.0 ± 74.0 g SD, and a mean GSI of 0.5 ± 1.0 SD while females (n = 15) had a mean SL of 10.8 ± 1.0 cm SD (range = 8.9–12.5 cm), a mean BM of 12.21 ± 4.16 cm SD, and a mean GSI of 0.9 ± 0.2 SD. Note that we could not calculate GSI in larvae, small juveniles, or large male juveniles because gonads were too small to weigh.

HC Bundle Density of Saccule

HC bundle density of the saccule varied by epithelial region, developmental stage (larvae, small juveniles, large juveniles, and adults), and by epithelial region across developmental stage (two-way mixed ANOVA with main effects of epithelial region [$F_{5, 260}$ = 65.15, $p < 0.05$] and developmental stage [$F_{3, 52}$ = 133.73, $p < 0.05$]; there was also a significant interaction of epithelial region and developmental stage [$F_{15, 260}$ = 9.25, $p < 0.05$]) (Fig. 1). HC density differed by developmental stage in region 1 ($F_{3, 52}$ = 26.18, $p < 0.05$), region 2 ($F_{3, 52}$ = 52.42, $p < 0.05$), region 3 ($F_{3, 52}$ = 18.31, $p < 0.05$), region 4 ($F_{3, 52}$ = 130.01, $p < 0.05$), region 5 ($F_{3, 52}$ = 105.00, $p < 0.05$), and region 6 ($F_{3, 52}$ = 69.64 $p < 0.05$) (one-way ANOVAs for simple main effects). There was also an effect of developmental stage (excluding larvae) on HC density in region 7 (one-way ANOVA, $F_{2, 48}$ = 78.34, $p < 0.05$). Larvae and small juveniles had greater HC bundle density than large juveniles and adults in regions 1, 2, 4, 5, and 6 (see Table 1 for regional HC density means; mean differences among the regions are based on post hoc pairwise comparisons with

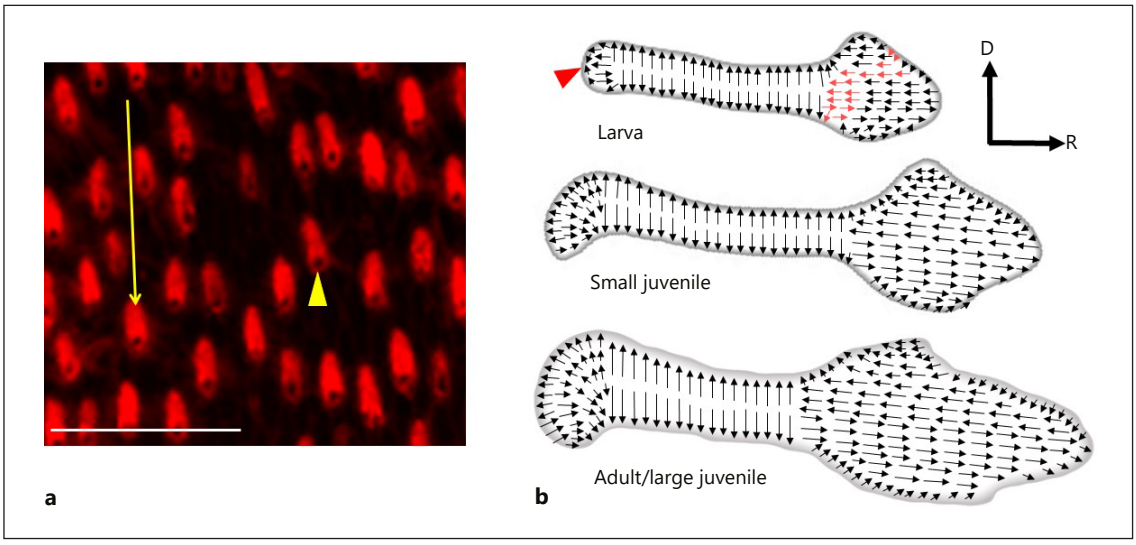

Fig. 3. HC orientation patterns. **a** Representative image of a juvenile's saccular macula. HC orientation was determined by visualizing the location of the kinocilium (yellow triangle) on the cuticular plate (yellow arrow). White scale bar, 25 μm. **b** HC orientation patterns in larvae (n = 10), small juveniles (n = 12), and adult/large juvenile saccular maculae (n = 4). Sketches of the maculae are not drawn to scale. The red arrows in the larval sketch were not visualized and are instead estimated HC orientation patterns based on the other developmental stages. D, dorsal; R, rostral.

Bonferroni corrections). In region 3, small juveniles had greater HC bundle density than large juveniles and adults, but larvae did not. There was no difference in HC bundle density between larvae and small juveniles in any examined regions except for region 4 in which larvae had greater HC bundle density than small juveniles. Similarly, HC bundle density was not different in adults and large juveniles in any examined regions. Small juveniles had greater HC bundle density than large juveniles and adults in region 7 (Fig. 1). In sum, larvae and small juveniles had greater HC bundle density than large juveniles and adults throughout most of the saccular epithelium, including both marginal/central and rostral/caudal zones.

Saccular Macula Shape and HC Orientation

The size (area) of the saccular macula increased with developmental stage from larvae to nonreproductive adults. Larvae had the smallest macular area (mean = 0.08 ± 0.02 mm^2 SD) followed by small juveniles (mean = 0.17 ± 0.02 mm^2 SD) and then large juveniles (mean = 1.22 ± 0.45 mm^2 SD) while the greatest macular area was observed in adults (mean = 2.64 ± 1.7 mm^2 SD) ($F_{3, 62}$ = 434.0, $p < 0.05$). Saccular macula size based on positional area (rostral, medial, and caudal) decreased in order from the rostral, medial, and caudal area in all developmental stages ($p < 0.05$) (Fig. 2).

The pattern of HC orientation in the midshipman saccule is most similar to the "standard pattern" described in other teleost fishes [Popper and Schilt, 2008], and this standard pattern of HC orientation was generally retained throughout saccular development. In the rostral zone HCs were oriented in a horizontal plane of maximum stereocilia depolarization. The border of the rostral and medial macula was defined by an abrupt 90-degree change in orientation as the stereocilia in the medial macula are oriented vertically in the z axis. In the caudal zone, the stereocilia gradually transition from a vertical orientation in the medial area of the macula to a horizontal orientation at the peripheral caudal end (Fig. 3). There were no obvious differences in saccule shape or HC orientation between large juveniles and adults (bottom diagram in Fig. 3; panel b represents both developmental stages). In addition, there were also no differences in HC orientation patterns between females and males (based on developmental groups where sex was known), therefore the sketches in Figure 3 represent data taken from both sexes.

Estimated HC Numbers in the Saccule, Lagena, and Utricle

The estimated number of HCs in the saccule increased with developmental stage from larvae to nonreproductive

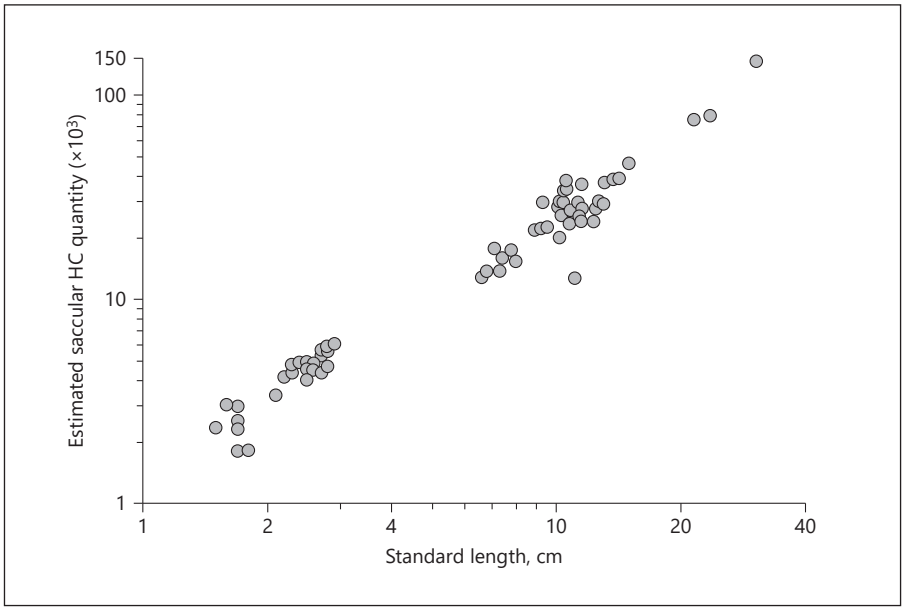

Fig. 4. Estimated saccular HC numbers. The estimated total number of saccular HCs increases as a power function of standard length during ontogeny from larvae to adults.

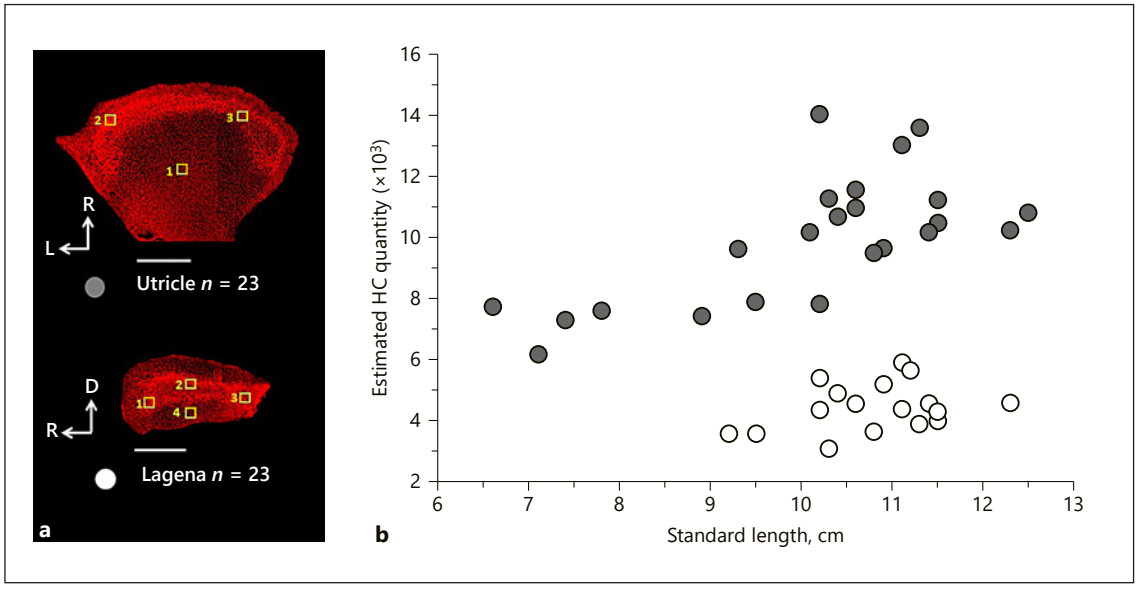

Fig. 5. Estimated HC numbers in the utricle and lagena. **a** Representative utricle (top) and lagena (bottom) maculae. Counts were made in 50 × 50 μm square areas with a total square area of 2,500 μm^2. Boxes are drawn to scale. R, rostral; L, lateral; D, dorsal; white scale bar, 250 μm. **b** Estimated HC numbers in the utricle and lagena increase as a power function of standard length. Gray circles, utricle; white circles, lagena.

adults. The estimated number of HCs in the saccule increased logarithmically during ontogeny as a nonlinear function of body size (SL) (estimated number of HCs = $1.24 \times (SL)^{1.3}$, $R^2 = 0.93$). The smallest larva (SL = 1.5 cm) had an estimated 2,336 HCs and the largest nonreproductive adult (SL = 30.5 cm) had the highest estimated number of HCs (145,717 HCs) (Fig. 4). The estimated number of HCs in the utricle and lagena also increased with developmental stage from large juveniles to nonreproductive adults. The estimated HC number in the utricle and lagena increased as a nonlinear function of body size in large juvenile and adult midshipman fish (estimated number of utricle HCs = $1.18 \times (SL)^{0.92}$, $R^2 = 0.55$; estimated number of lagena HCs = $0.57 \times (SL)^{0.86}$, $R^2 = 0.12$)

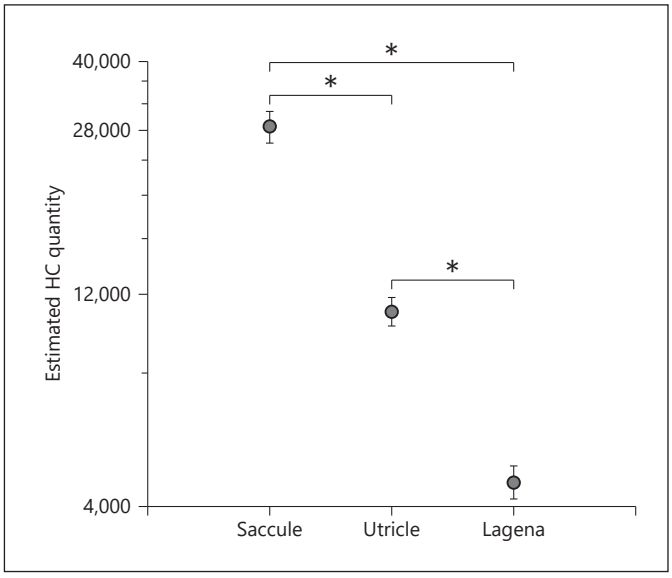

Fig. 6. Estimated HC numbers of the saccule, utricle, and lagena in the adult midshipman. Estimated HC numbers were highest in the saccule, intermediate in the utricle, and lowest in the lagena. Asterisks denote statistically significant differences in Bonferroni pairwise comparisons ($p < 0.05$). Error bars represent ±95% confidence intervals.

(Fig. 5). The estimated HC numbers varied among inner ear end organs in nonreproductive adults ($F_{2, 44} = 502$, $p < 0.05$) with the saccule having the highest estimated number of HCs (mean = 28,479 ± 4,809 SD) followed by the utricle (mean = 11,008 ± 1,619 SD) and then the lagena (mean = 4,560 ± 769 SD), which had the lowest estimated number of HCs ($p < 0.05$) (Fig. 6).

Discussion

The primary aim of this study was to characterize the ontogenetic saccular development in the plainfin midshipman and determine whether the density, total number, and orientation patterns of HCs in the saccule change during ontogeny from larvae to nonreproductive adults. A secondary aim was to determine whether the total number of HCs in the saccule differed from that of the utricle and lagena in nonreproductive adults. We found that saccular HC density varied across developmental stage with larvae and small juveniles having greater HC bundle density than large juveniles and adults in most regions. The ontogenetic reduction in HC density was concurrent with an increase in macula area. The orientation pattern of saccular HCs was similar to the standard pattern previously described in other teleost fishes, and this pattern of HC orientation was retained during ontogeny. Lastly, the estimated number of saccular HCs increased with developmental stage from larvae to nonreproductive adults, and in nonreproductive adults estimated HC numbers were highest in the saccule, intermediate in the utricle, and lowest in the lagena. In this discussion, we interpret our results as they relate to the physiology of the auditory inner ear end organs.

The observed decrease in midshipman saccular HC density with developmental stage from larvae and small juveniles to large juveniles and nonreproductive adults was similar to an observed decrease in HC density throughout development in other teleost fishes including the oscar cichlid (*Astronotus ocellatus*), European hake (*Merluccis merluccius*), and the Lusitanian toadfish (*Halobatrachus didactylus*) [Popper and Hoxter, 1984; Lombarte and Popper, 1994, 2004; Chaves et al., 2017], with the exception of zebrafish (*Danio rerio*) where HC density increased from small juveniles to young adults and then decreased in older adults [Wang et al., 2015]. In addition, we showed that the area of the saccular macula in the midshipman increased with developmental stage in a nonlinear manner from larvae (mean = 0.08 mm^2) to small juveniles (mean = 0.17 mm^2) to large juveniles (mean = 1.22 mm^2) and then to adults (mean = 2.64 mm^2). This nonlinear increase in saccular macula area during midshipman development coupled with the concurrent addition of new HCs in the growing macula likely contributes in part to the observed decrease in mean saccular HC density during ontogeny.

We also found that the HC orientation patterns in the midshipman saccule were similar to the "standard" pattern described by Popper [1981] and this observed pattern was generally retained during ontogeny in the midshipman. This standard HC orientation pattern is generally found in species that lack an otophysic connection [Popper, 1977, 1981; Popper and Schilt, 2008]. In addition, the HC orientation pattern and overall shape of the midshipman saccular macula also resembled that found in the closely related Gulf toadfish (*Opsanus beta*), Oyster toadfish (*Opsanus tau*), and Lusitanian toadfish [Popper, 1981; Edds-Walton and Popper, 1995; Chaves et al., 2017]. Interestingly, we observed an ontogenetic change in the orientation of HCs at the marginal end of the caudal macula in larvae (red arrowhead, Fig. 3) that changed from vertically oriented HCs to horizontally oriented HCs that followed the macula margin in juveniles and adults. Recently Colleye et al. [2019] have shown that female midshipman fish which possess sexually dimorphic rostral swim bladder extensions have enhanced auditory sensitivity to sound pressure and fre-

quencies >305 Hz. The swim bladder horn-like extensions decrease the distance between the swim bladder and saccule to effectively enhance the detection of local particle motion generated by pressure-induced vibrations of the swim bladder when exposed to sound. Thus, the swim bladder in females and type II males, which also possess rostral swim bladder extensions [Mohr et al., 2017], is thought to serve as an acoustic organ that enables the indirect detection of sound pressure stimuli needed for conspecific localization and social signal detection of conspecifics. The observed ontogenetic enlargement of the caudal and rostral areas of the saccular macula, which contain primarily horizontally oriented HCs, may play an important role in the detection of sound pressure-induced vibrations of the swim bladder in juveniles and adults, especially in females and type II males. It still remains unclear whether type I males, which do not possess the rostral horn-like swim bladder extensions, are able to detect the pressure-induced vibrations of the swim bladder. Future work that investigates the local particle motion and directional movement of the sagitta (saccular otolith) in response to the sound pressure wave-induced vibrations of the midshipman swim bladder will be informative in determining the importance and function of the horizontally oriented HCs in the caudal and rostral areas of the saccule during development.

Our results revealed that the estimated number of HCs in the saccule increased during ontogeny as a nonlinear function of body size (SL) and that in nonreproductive adults the estimated number of HCs in the three midshipman auditory end organs varied with the saccule having the greatest number of HCs. The estimated ontogenetic increase in HC number was greater than that reported for the Lusitanian toadfish (*Halobatrachus didactylus*) [Chaves et al., 2017]. In the midshipman, we report an ontogenetic increase in the estimated number of saccular HCs from 2,336 HCs in the smallest larvae to 145,717 HCs in the largest nonreproductive adult, whereas Chaves et al. [2017] reported an ontogenetic increase in the estimated number of saccular HCs from 1,247 HCs in larvae to 31,616 HCs in adults. In addition, we found that the estimated number of HCs varied among the different midshipman end organs. In nonreproductive adults, the mean HC number in the saccule (mean = 28,479 HCs) was 2.6 times greater than that in the utricle (mean = 11,008 HCs) and 6.2 times greater than in the lagena (mean = 4,560 HCs). These end organ differences in HC number are likely related to the end organ size of the macular area with the saccule having the largest macular area in general (note, we did not compare the macular areas of the utricle, lagena, and saccule, but the differences in end organ size visually are obvious). Not surprisingly, the lagena, which is the smallest end organ, is significantly less sensitive than the saccule and utricle based on HC auditory evoked potentials [Colleye et al., 2019; Vetter et al., 2019; Rogers and Sisneros, 2020]. In addition to having fewer total HCs as we report here, the lagena also has the lowest otolith mass (smallest of the three end organs) and contains an otolith (asteriscus) that is predominantly composed of lighter vaterite [Campana, 1999; Reimer et al., 2016]. Although the adult utricle has a significantly lower number of HCs than the saccule, a recent study by Rogers and Sisneros [2020] showed that the auditory sensitivity of the utricle based on HC auditory evoked potentials was very similar to the saccular sensitivity of type I males at frequencies <305 Hz; however, at frequencies >305 Hz the utricle was even more sensitive than the saccule [Colleye et al., 2019]. Given that the otoliths of the saccule (sagitta) and utricle (lapillus) are both primarily composed of calcium carbonate in the form of aragonite [Thorrold and Hare, 2002] and would share similar densities but different overall otolith masses, it remains unclear how differences in otolith mass and end organ HC numbers and densities would affect auditory sensitivity based on HC auditory evoked potentials.

Although ontogenetic development of the inner ear in fishes is well documented [Popper and Hoxter, 1984; Sokolowski and Popper, 1987; Popper and Hoxter, 1990; Vasconcelos et al., 2015] less is known about how the development of the inner ear affects auditory sensitivity in fishes during ontogeny. In the thornback ray (*Raja clavata*), the total number of HCs in the macula neglecta, a nonotolithic detector of sound in elasmobranchs, was found to increase during development and was concurrent with increased afferent sensitivity of the macula neglecta [Corwin, 1983]. In contrast, Higgs et al. [2002] showed that the zebrafish (*Danio rerio*) exhibited no change in auditory sensitivity based on auditory evoked potentials with ontogenetic increases in total HC number in the saccule, the main organ of hearing in the zebrafish and most teleost fishes. However, Wang et al. [2015] showed a concurrent decrease in zebrafish auditory evoked potential thresholds (i.e., increased auditory sensitivity) with ontogenetic increases in saccular HC density and saccular HC numbers. Finally, Vasconcelos et al. [2015] showed that the Lusitanian toadfish (*Halobatrachus didactylus*) exhibited developmental stage-dependent increases in auditory saccular sensitivity based on saccular potential recordings as toadfish transitioned from juveniles into adults, which is likely, in part, due to the observed ontogenetic increases in total HC number in the toadfish saccule [Chaves et al., 2017].

In the current midshipman study, we showed that saccular HC density varied across developmental stage with larvae and small juveniles having greater HC bundle density than large juveniles and adults in most saccular regions. It remains unclear how HC density of the saccule influences saccular potential sensitivity measurements in the midshipman and the related Lusitanian toadfish. Previous ontogenetic studies of peripheral auditory sensitivity in the plainfin midshipman fish have yielded conflicting results in terms of how peripheral auditory sensitivity changes during ontogeny. Alderks and Sisneros [2011] showed that the auditory threshold tuning curves based on auditory evoked saccular potentials did not change during ontogeny from small juveniles to large juveniles to adults for frequencies that ranged from 75 to 785 Hz. However, Sisneros and Bass [2005] showed that based on saccular afferent recordings auditory sensitivity increased with size/age from small juveniles to adults. These results are different from the results for the auditory evoked HC potential measurements and suggest that midshipman fish do exhibit ontogenetic increases in auditory sensitivity at least at the level of auditory afferents postsynaptic to the HCs. Changes in saccular afferent sensitivity may be related to ontogenetic changes in the convergence ratio of HCs to auditory afferents during ontogenetic saccular development. Future studies that examine ontogenetic changes in HC-afferent convergence ratio coupled with an examination of ontogenetic changes in otolith mass will be instrumental in helping to determine how other changes in the saccule besides HC number and density contribute to ontogenetic changes in the auditory sensitivity of the midshipman saccule.

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Statement of Ethics

All experimental procedures conformed to NIH guidelines for animal care and use of animals and were approved by the University of Washington Institutional Animal Care and Use Committee (Protocol 4079-01).

Conflict of Interest Statement

The authors have no conflicts of interest to declare.

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Author Contributions

N.R.L., J.A.S.: conceptualization, methodology, funding acquisition; N.R.L.: data curation, investigation, formal analysis, visualization, writing – original draft; J.A.S.: supervision, project administration, writing – review and editing.

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