RESEARCH ARTICLE

Reproductive-state dependent changes in inner ear gene expression in female plainfin midshipman fish

Coty Jasper[1¤a], Sumaya Hassan[2], Robin Gibson[2], Tot Nguyen[2], Olivia Molano[3¤b], Leila Farbod[3], Joseph A. Sisneros[4], Jennifer S. Stone[2], Allison B. Coffin[1,3,5]*

1 Department of Integrative Physiology and Neuroscience, Washington State University, Vancouver, Washington, United States of America, **2** The Virginia Merrill Bloedel Hearing Research Center and the Department of Otolaryngology-Head and Neck Surgery, University of Washington, Seattle, Washington, United States of America, **3** Neuroscience Program, College of Arts and Sciences, Washington State University, Vancouver, Washington United States of America, **4** Department of Psychology, University of Washington, Seattle, Washington, United States of America, **5** Bellucci Translational Hearing Center and Department of Biomedical Sciences, Creighton University, Omaha, Nebraska, United States of America

¤a Current address: Medical Research Institute of Chemical Defense, Aberdeen Proving Ground, Maryland, United States of America.
¤b Current address: Section on Sensory Cell Development and Function, National Institute on Deafness and Other Communication Disorders, Bethesda, Maryland, United States of America.
* allisoncoffin@creighton.edu

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Abstract

Acoustic communication is vital for reproductive success in many vertebrates. In the plainfin midshipman fish (*Porichthys notatus*), females respond to low-frequency advertisement calls ("hums") produced by nesting type I males during the summer reproductive season, likely driving mate selection. Females exhibit seasonal, estrogen-mediated auditory plasticity that adaptively couples the sender and receiver to enhance reproductive success. Physiological plasticity in two regions of the inner ear, the saccule and the utricle, increases sensitivity to key features of the males' advertisement call in reproductive female midshipman. However, the mechanisms underlying this plasticity differ between inner ear regions. In the saccule, enhanced encoding of the male's call is correlated with an increase in cell proliferation and the number of sensory hair cells. By contrast, plasticity in auditory sensitivity in the utricle is independent of cell addition. In this study, we used nCounter technology to determine the transcriptional changes associated with auditory plasticity in the female midshipman inner ear. We found that the transcriptional profile of the saccule was distinct from the utricle. More of our target genes were differentially expressed in the saccule than the utricle, and more saccular transcripts showed seasonal changes in gene expression as compared to the other inner ear regions. Our results offer new insights into how gene expression differentially regulates auditory plasticity in the female midshipman inner ear and provides a framework for future studies of hormonally-mediated plasticity in other vertebrates.

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Introduction

Acoustic communication plays a vital role in courtship and reproductive success of many vertebrate species. For example, in songbirds such as the Carolina chickadee (*Poecile carolineses*) and the tufted titmouse (*Baeolophus bicolor*), males produce vocalizations to attract females during the breeding season [1]. These behaviors are accompanied by seasonal changes in auditory sensitivity, which are thought to enhance detection of courtship signals when reproduction is mostly likely to occur [1]. Similar patterns of seasonal auditory plasticity are observed in the plainfin midshipman fish (*Porichthys notatus*, here referred to as midshipman), a soniferous teleost fish native to the Pacific coast of North America. Like songbirds, midshipman rely on acoustic signaling for mate attraction and show hormonally mediated changes in auditory sensitivity that coincide with breeding status [2–4].

During the summer breeding season, large "Type I" male midshipman establish nests under intertidal rocks and produce low-frequency advertisement calls ("hums") to attract gravid females for reproduction [4–6]. Playback experiments have shown that reproductive-state females are strongly attracted to these courtship hums, indicating that acoustic cues are a primary signal for mate detection and localization [5,7]. Neurophysiology studies demonstrate that key features of the male hum are encoded in the peripheral auditory system and that these encoding features are enhanced during the breeding season [8–11]. For example, reproductive females exhibit increased phase locking in the 8th cranial nerve, enabling more precise encoding of the periodicity in male hums [8]. Additionally, the auditory end organs of the inner ear, the saccule and the utricle, show seasonal shifts in auditory sensitivity, although no shift in auditory sensitivity has been observed within the lagena, an inner ear end organ that may be important for detecting higher intensity signals such as nearby conspecific vocalizations [12]. Specifically, auditory thresholds in the saccule and utricle are lower (*i.e.,* sensitivity is increased) in reproductive females compared to their non-reproductive counterparts [8,13].

These functional changes are supported by underlying morphological plasticity. The saccule of reproductive females contains a higher density of sound-transducing hair cells, a change not observed in the utricle or lagena [14]. This seasonal increase in hair cell density suggests that structural remodeling of the inner ear contributes to auditory enhancement. Together, these findings point to multiple mechanisms–neural, physiological, and cellular–that underlie reproductive state-dependent changes in auditory processing in the midshipman fish. However, the transcriptional mechanisms that regulate these seasonal changes remain largely unknown.

The goal of our study is to identify gene expression changes in the female midshipman inner ear that correspond with physiological and morphological differences observed between reproductive and non-reproductive states. Prior pharmacological studies have shown that manipulating specific signaling pathways can reverse reproductive-state enhancements in auditory sensitivity [15,16]. For instance, blocking large-conductance calcium-activated potassium (BK) channels or activating D2A dopamine receptors in reproductive midshipman females results in elevated saccular auditory thresholds, effectively reverting to non-reproductive-like auditory phenotypes

[15,16]. These findings suggest that BK channel activity and dopamine signaling contribute to auditory plasticity and may be regulated at the transcriptional level. Based on this evidence, we hypothesize that reproductive females will show increased BK channel gene expression and decreased dopamine receptor gene expression in the saccule and utricle.

In addition to these functional changes, the observed increase in saccular hair cell density during the breeding season implies modulation in rates of hair cell turnover (loss and replacement) at distinct phases throughout the year. These changes could occur through increased progenitor (supporting) cell proliferation, hair cell precursor differentiation, reduced hair cell death, or a combination of these processes. A previous RNA-seq analysis of female midshipman inner ears indicated reproductive state-dependent shifts in the expression of genes in the Wnt, Notch, and heat shock protein (Hsp) pathways [17], which regulate cell proliferation, differentiation, and/or survival in hair-cell epithelia. For example, Wnt signaling is required for normal levels of hair cell addition in the developing basilar papilla of chickens and in regenerating lateral line neuromasts of zebrafish [18–20], while Notch signaling promotes supporting cell division and/or suppresses hair cell addition in avian inner ear organs [18,21,22]. Furthermore, Hsp genes support hair cell survival under conditions of cellular stress, such as noise exposure or chemical insult, in the mammalian cochlea and utricle [23,24].

Based on these data, we hypothesized that the saccule of reproductive females will exhibit increased expression of Wnt and Hsp genes and decreased expression of Notch pathway components, patterns not expected to be present in the utricle, which does not show seasonal increases in hair cell density. To test this hypothesis, we quantified gene expression in inner ear end organs from reproductive and non-reproductive female midshipman using nCounter transcript analysis. We elected to use nCounter technology to quantify transcriptional changes in specific pathways of interest governing cell addition, cell survival, and hair cell tuning. Further, we analyzed each inner ear end organ (lagena, saccule, and utricle) separately because different cellular mechanisms likely drive physiological plasticity in an end organ-specific manner. This study complements our previous RNA-Seq study, which used whole inner ear rather than isolated end organs [17]. Our results reveal seasonal and end-organ specific transcriptional differences that align with previously characterized physiological and morphological changes in midshipman auditory sensitivity. This study provides new insight into the molecular mechanisms that support seasonal auditory plasticity in the context of hair cell addition in a vertebrate model system, with potential implications for understanding the genetic regulation of sensory plasticity more broadly.

Materials and methods

Fish collection

Non-breeding state female midshipman (also called non-reproductive or winter fish) were collected in January 2023 via otter trawl in Monterey Bay, California (USA) and transported to the Coffin Lab at Washington State University, Vancouver (WSUV). Non-reproductive fish were given a two-week acclimation period prior to experimentation to recover from the stress of capture at depth and transportation. Fish were housed at 14–16 °C on an 8/16 light/dark cycle in 32 ppm salt water. Breeding-state females (also referred to as reproductive or summer fish) were collected by hand from the Hood Canal shoreline in Washington State (USA) during the summer months (May to July, 2023). Midshipman nests were accessed underneath rocks in the intertidal zone during low tide. Females were gathered directly from nests and transported to WSUV within three hours of collection. Summer fish were euthanized within 6–8 hours of collection (shortly after transport to the research facility) because they were not exposed to the same stressful pressure changes that winter fish encountered during their collection.

For all animals we measured standard length (nose to base of caudal fin), fish mass, and gonad mass. We then calculated the gonadal somatic index (GSI, calculated as 100 * gonad mass/(body mass – gonad mass)), which serves as a measure of relative reproductive state [25].

All fish collections were authorized by U.S. Fish and Wildlife under permit number SISNEROS 23–215. All procedures were approved by the Institutional Animal Care and Use Committee of Washington State University under protocol 6194.

Tissue dissection and gene expression analysis

mRNA sequencing. Fish were euthanized with Syncaine MS-222 (150−200 mg/L, Pentair Aquatic Eco-systems). Sensory end organs (saccules, utricles, and lagenae) were dissected from the head, then otoliths and non-sensory regions of the inner ear were trimmed away, leaving the epithelium and a portion of the attached innervation We also dissected out gill tissue as a non-auditory control. Ear and gill tissue was preserved in RNAlater (ThermoFisher Scientific) and stored at −20 °C prior to RNA extraction using an RNeasy Mini kit (Qiagen). Samples were further purified with an RNA Clean & Concentrator-5 kit (Zymo Research). Individual inner ear organs did not yield enough RNA for analysis (unpublished observation), so lagenae, utricles, saccules, or gills were pooled using tissue from three fish per group to yield four pooled samples per tissue and season. RNA samples were then transported on dry ice (−80 °C) to Washington State University's genomics core in Pullman, Washington.

Transcript levels were quantified via NanoString nCounter analysis using a custom probe set for the 37 genes that we selected to examine (see S1 Table) and analyzed using nSolver software. The nCounter process first hybridizes RNA samples to fluorophore-labelled probes specific for each gene. Positive control probes were also included as measured of hybridization efficiency, as were negative control probes that should not hybridize [26]. Probes and controls were optically counted to quantify total transcript number of each gene and estimate type I and type II error rates [26]. To account for potential variation in expression levels in our pooled samples, including variation in normalization probes, we utilized the geNorm algorithm using the ctrlGene R package to select genes to normalize expression [27]. The genes *ctnnb1* (which encodes β-catenin), *gsk-3α*, and *hsbp-1* had the highest ratio of gene expression-to-variance across all auditory tissues and were selected as normalization genes [28]. Data were additionally normalized (discussed below) with positive controls of synthetic ssDNA of known counts added to each sample. Normalization was conducted with the nSolver software for both positive controls and our selected normalization genes. Briefly, a normalization factor was derived by calculating the geometric mean of all positive controls from a sample, calculating the arithmetic mean of all geometric means across samples, and finally dividing the arithmetic mean by the geometric mean of a sample. Raw counts were then normalized by multiplying counts with normalization factors from positive controls and normalization genes [27].

mRNA localization in inner ear organs. Fish were euthanized as described above. Saccules were removed from the head and immersion-fixed for 2–3 hours at room temperature in 4% buffered paraformaldehyde. Following rinses in phosphate buffered saline (PBS, ThermoFisher Scientific) with 0.1% Tween20 (Millipore Sigma) dissolved in PBS (PBT), end organs were dehydrated in a gradient series of methanol. Tissue was stored in 100% methanol at −80 °C for periods ranging from 2–24 months prior to *in situ* detection of mRNAs using the hybridization chain reaction (HCR).

Saccules were embedded in agarose (3%, dissolved in sterile PBS, Promega), and 30–50 μm vibratome sections were generated under sterile conditions. Sections were processed for HCR on the same day, following instructions from the manufacturer (Molecular Instruments). Using a 96-well plate, tissue sections were treated with proteinase K (10 μg/ml) for 10 min at room temperature, rinsed with PBT, fixed for 20 min in 4% formaldehyde/0.1% glutaraldehyde in PBT, rinsed, and hybridized overnight at 37 °C. HCR probes targeting genes of interest were designed using contig sequence information from our RNA-seq dataset (*jag1b* c43669 (210 nt), *lef1* c63110 (395 nt), *fzd7a* c43974 (1–240), *hsp70* c22598 (323 nt) [17]. For *deltaA* (AZ), HCR probe was designed using sequence for *Thalassophryne amazonica* XM_034187904.1. Pre-amplification and amplification steps were carried out at 37 °C. Following HCR, tissue sections were co-labeled with 4′,6-diamidino-2-phenylindole (DAPI, from Millipore Sigma) to define cell nuclei. Some sections were incubated overnight at room temperature in rabbit anti-myosin VIIa IgG (Invitrogen, formerly Proteus) diluted 1:300 in 10% normal horse serum in PBS. The next day, sections were rinsed in PBS, then incubated in 1:500 Alexa Fluor 488 conjugated donkey anti-rabbit IgG (Life Technologies) for 2–3 hours at room temperature, rinsed again in PBS, and mounted on microscope slides in ProLong™ Gold anti-fade reagent (Invitrogen) with a coverslip.

We examined HCR and immunofluorescence labeling in saccule sections using an Olympus FV1000 confocal microscope, generating Z-stacks using 20x and 60x objectives. We used FIJI software (imagej.net) to adjust and process images, generating files that were further processed using Photoshop (Adobe) (San Jose, CA).

Statistical analysis

We used the nSolver software to read and standardize our nCounter data for gene expression analysis as described above. The nSolver algorithm was used to perform log-linear modelling of gene expression between seasons and to derive P values using season as an identifier, with GSI and standard length as covariates. We used principal component analysis (PCA) and Benjamini-Yekutieli false discovery rate post-hoc testing within the nCounter software to determine how standard length and GSI affected the variability within our gene expression data. The nSolver normalization algorithm also accounts for transcript count variation between samples. PCA analysis was conducted using R v. 4.2.0. To visualize gene expression patterns across samples and seasons, we generated heat maps of gene expression for each sample from each tissue using the pheatmap R package on extracted normalized data from the nSolver analysis.

We also wanted to observe differences in the magnitude of gene expression changes between the saccule and utricle. Raw data from nSolver was imported into R and normalized using the DESeq2 R package, which uses maximum likelihood methods to estimate dispersion based on average gene expression values within a dataset [29]. We then used the DESeq2 package to perform a differential expression analysis between utricles and saccules from each season and generated volcano plots of the results using the ggplot2 package. We used threshold annotations of 2- and 1.5-fold change, as there is no universal standard by which a given fold-change differences may be considered biologically meaningful [27,30]. All nCounter datasets reported in this publication are available on Mendeley Data (https://doi.org/10.17632/grn429gvgx.1). Normalized gene expression data and associated statistics are located in supplemental S1 File.

Results

Variation in body length and GSI across season

We first compared body length and gonadosomatic index (GSI) between seasonal sample populations of midshipman. Within our pooled samples standard length was 11.02 ± 1.60 cm (Mean ± SD) in non-reproductive fish and 17.10 ± 1.70 cm in reproductive fish (t-test, $p < 0.0001$), while GSI was 3.01 ± 2.99 and 14.71 ± 5.90 in non-reproductive and reproductive fish, respectively (t-test, $p < 0.0001$). Because of the seasonal differences in mean GSI and standard length, these metrics were incorporated as covariates in the nSolver linear regression analysis to account for their potential influence on gene expression.

Overview of analytical approach

We next performed a principal component analysis (PCA) on normalized expression data to visualize how gene expression was influenced by tissue type (gill vs. auditory end organs). We then used heat maps to visualize the seasonal changes in gene expression patterns within each tissue and to show the variability between RNA samples. Finally, we quantified seasonal changes in gene expression within each inner ear organ, then compared seasonal differences between the saccule and utricle to assess how reproductive state correlates with known patterns of morphological and physiological auditory plasticity.

Gene expression patterns differ across seasons and tissues

PCA revealed clear segregation of gill versus inner ear samples, independent of season (Fig 1). Within the inner ear tissue cluster, the saccule grouped separately from the lagena and the utricle, while the lagena and utricle clustered closely

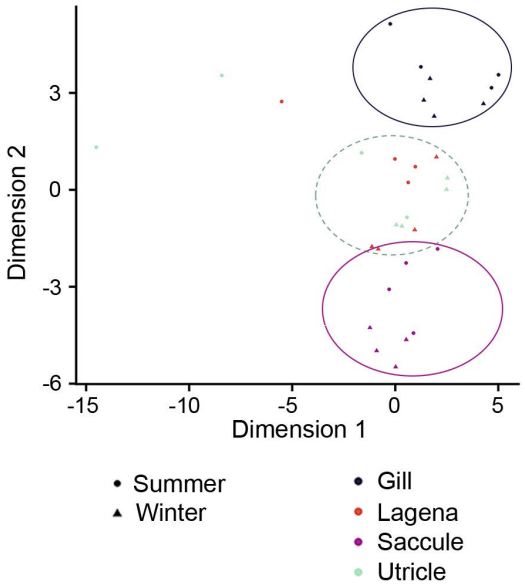

Fig 1. Inner ear and gill tissue show distinct gene expression patterns. Principle component analysis of gene expression comparing tissues and seasons. Gill tissue clustered separately from inner ear tissue. The saccule clusters separately from the utricle and lagena. Individual dots represent pooled samples (see Materials and methods). Color denotes tissue type, shape represents season. Gill datapoints are clustered higher on dimension two (top circle, black) while the saccule clusters at the bottom of dimension two (bottom circle, purple), with the utricle and lagena in an intermediate position (middle circle, green dotted line).

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together (Fig 1). These patterns demonstrate that auditory end organs possess gene expression profiles distinct from non-auditory tissue and suggest organ-specific transcriptional profiles within the inner ear.

Heat maps of gene expression within each tissue show clusters of up- and downregulated genes across seasons and illustrate between-sample variability (Fig 2). Gene expression patterns show less between-sample homogeneity for the gill and lagena; the two tissues that we expected not to show seasonal differences associated with reproductive state. By contrast, patterns in the utricle were relatively similar between samples for a given season and saccular samples showed some similarity, particularly in the summer. The pattern is particularly striking in the utricle, where the majority of transcripts examined are downregulated in all four utricular samples from non-reproductive fish. However, only two of the four utricle samples from reproductive (summer) females show marked upregulation of these transcripts (Fig 2). Notably, patterns of gene expression did not appear to be driven by a single sample across tissues. For example, summer samples 2 and 4 had high numbers of increased transcript counts in the utricle, while summer samples 1–3 had similarly low levels of gene expression in the saccule. These results suggest that while there is sample-to-sample variability, the results we describe below are not driven by a single sample but instead are representative of the larger dataset.

Seasonal stability of gene expression in gill tissue

To determine whether reproductive-state effects on gene expression extend to non-auditory tissues, we quantified transcript abundance in gills. Gene expression was seasonally stable for 34 of 37 genes (Fig 3). In reproductive females, however, the Notch ligand *deltaC* was significantly upregulated, whereas the estrogen receptor *era* and the Wnt pathway gene *gsk3b* were significantly downregulated relative to winter non-reproductive females. No seasonal differences were detected for genes associated with heat shock signaling, dopamine receptors, K^+ channels, or Wnt ligands. These results indicate that gill tissue exhibits minimal seasonally plasticity; therefore, the seasonal changes described below for inner ear tissues are likely inner ear-specific and associated with reproductive state.

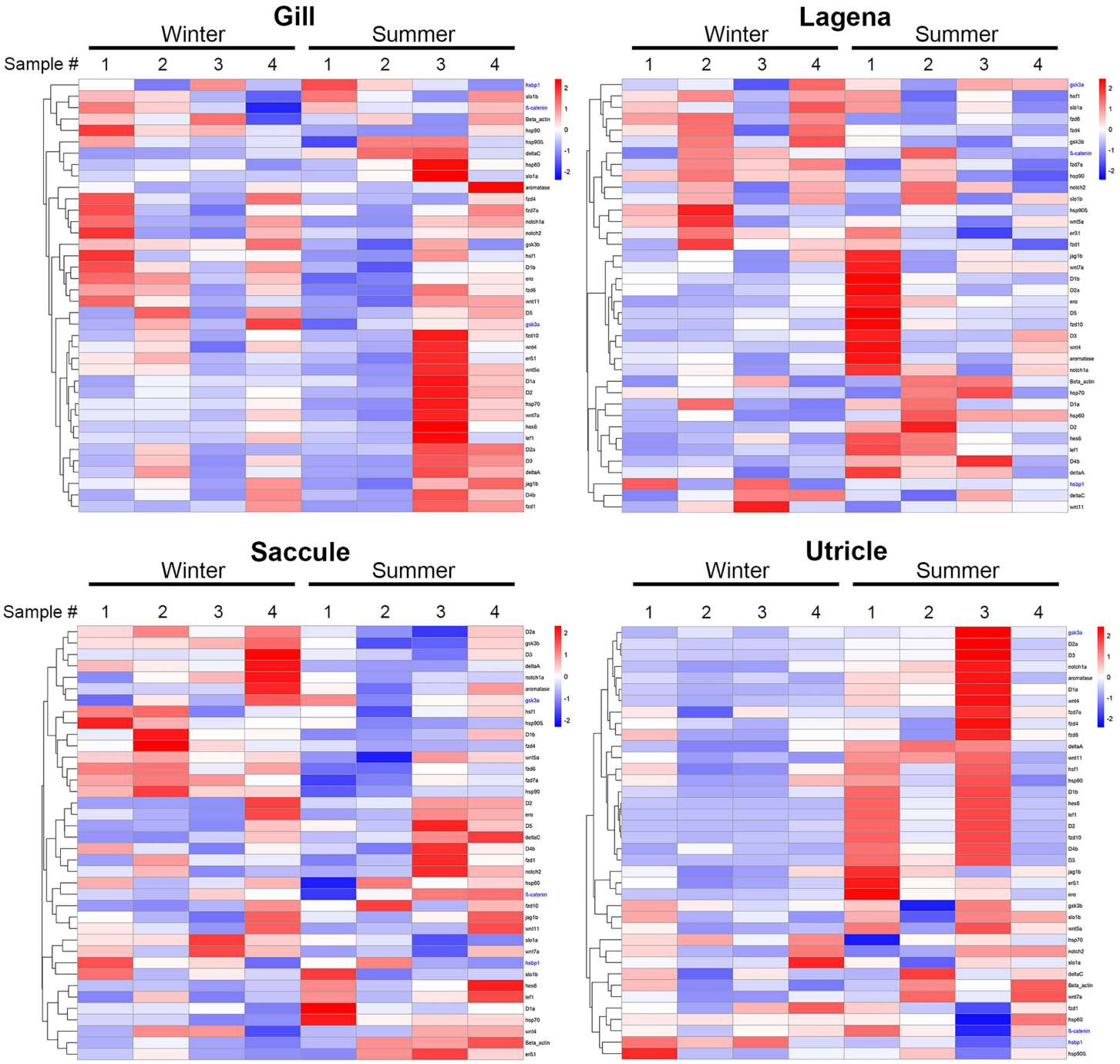

Fig 2. Heatmaps of gene expression by tissue and season. Each heat map shows the relative upregulation (red) or downregulation (blue) of normalized transcript levels within a tissue. For each figure, the four columns on the left represent the independent biological replicates from winter animals, while the four columns on the right represent the replicates from summer. Biological replicates are indicated as sample # 1-4 for each tissue and season.

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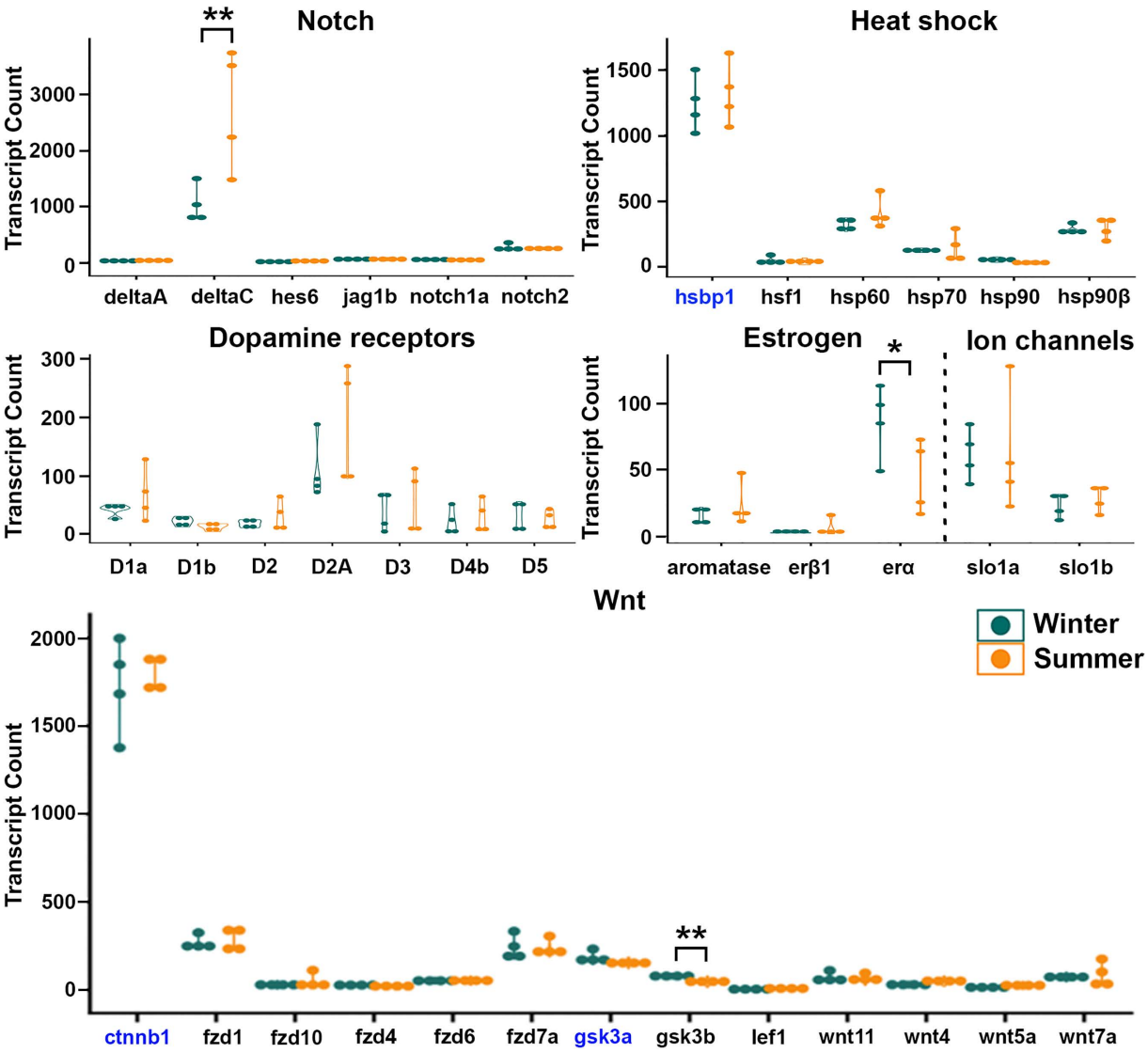

Fig 3. Gene expression in gills is generally stable between seasons. Quantification of gene expression from winter and summer female gills. A loglinear model was used to calculate linear regression scores. Only 3 of 37 genes had significantly different expression levels between seasons. In summer (reproductive) tissue expression of *deltaC* was significantly increased, while *erα* and *gsk3b* were significantly decreased, as compared to winter (non-reproductive) samples. *gsk3a, ctnnb1*, and *hsbp1* were used as normalization genes (blue). Data are presented in violin plots to represent the data structure; dots represent individual (pooled) samples, *$p < 0.05$, **$p < 0.01$.

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Seasonal gene expression in auditory end organs

We compared organ-specific gene expression profiles between reproductive and non-reproductive females. Overall, gene expression patterns were broadly similar across the three inner ear organs (Figs 4–6). For example, the lagena and saccule showed relatively similar expression patterns of the Notch ligands *jag1b* and the Notch receptors *notch1a* and *notch2,* irrespective of season, where *jag1b* and *notch2* were both expressed at higher levels and with more variability relative to *notch1a* (Figs 4–5). Similarly, we did not see seasonal or organ-specific biologically meaningful differences in expression of any Wnt ligand (*wnt4, 5a, 7a,* or *11*) (Figs 4–6). By contrast, *hsp70* expression was significantly elevated in

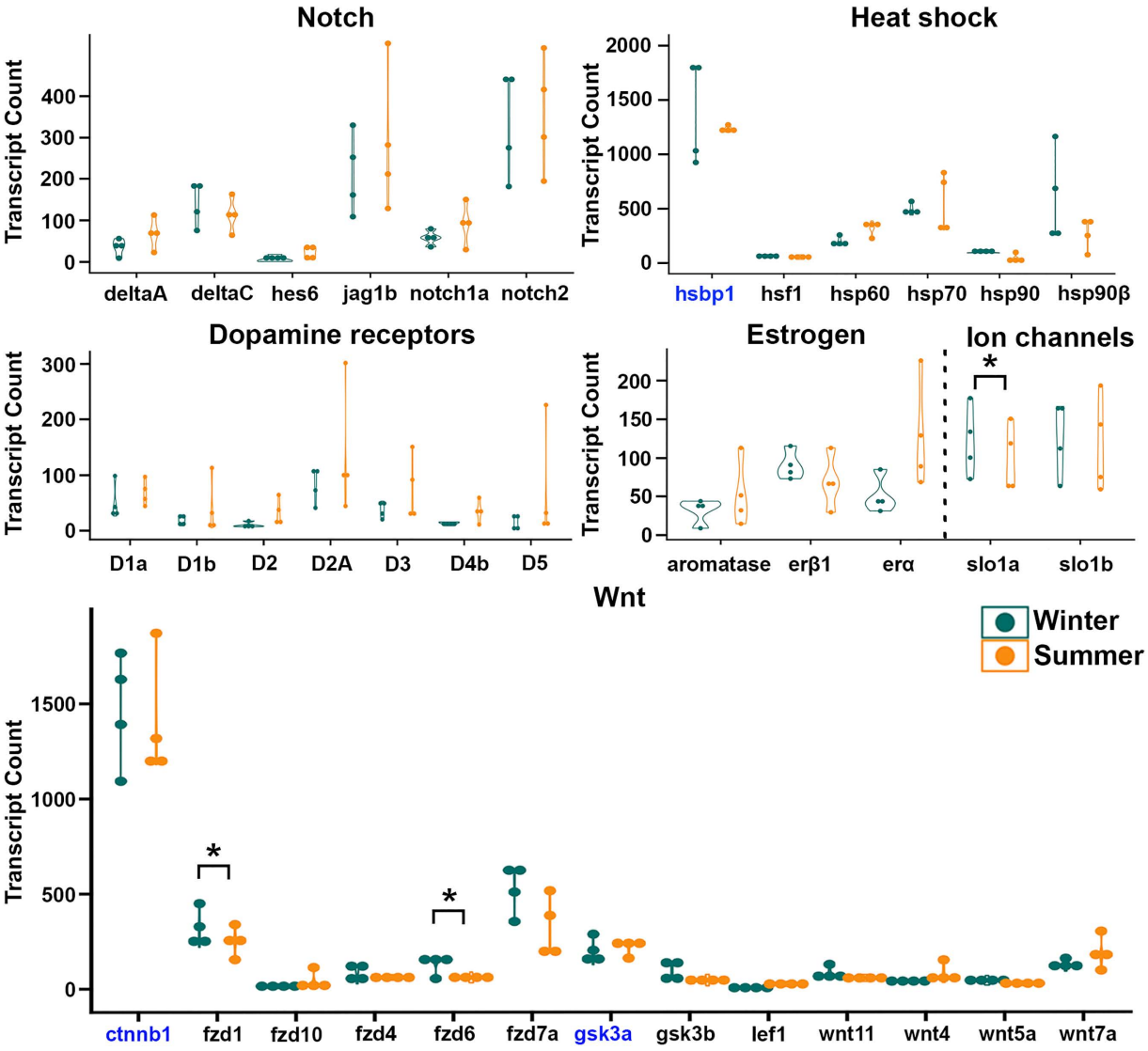

Fig 4. Gene expression in the lagena is relatively stable between seasons. Expression of three genes significantly differed between seasons, with *slo1a*, *fzd1,* and *fzd6* all significantly downregulated in lagenae from summer fish as compared to winter. *hsbp1*, *ctnnb1*, and *gsk3a* were used as normalization genes (blue). Dots represent individual (pooled) samples, *$p < 0.05$.

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saccules from reproductive females (Fig 5), and the BK channel gene *slo1a* was significantly upregulated in winter in all three sensory organs. Dopamine receptor expression was highly variable among organs, a pattern that may contribute to regional differences in auditory sensitivity within the inner ear [15,31]. Lastly, we identified several genes with clear organ-specific seasonal shifts in expression, which are detailed below.

There are no reported seasonal differences in hair cell density or physiological plasticity in the lagena of female midshipman [9,13,14]. In this context, this tissue represents baseline gene expression for the midshipman inner ear. Overall, the lagena had seasonally stable gene expression for 34 of 37 genes, consistent with our hypothesis that gene expression in this tissue is not substantially influenced by breeding state (Fig 4). Dopamine receptors were expressed at relatively low levels in non-reproductive tissue (normalized transcript counts 3.8–108.1) and displayed greater variability in reproductive

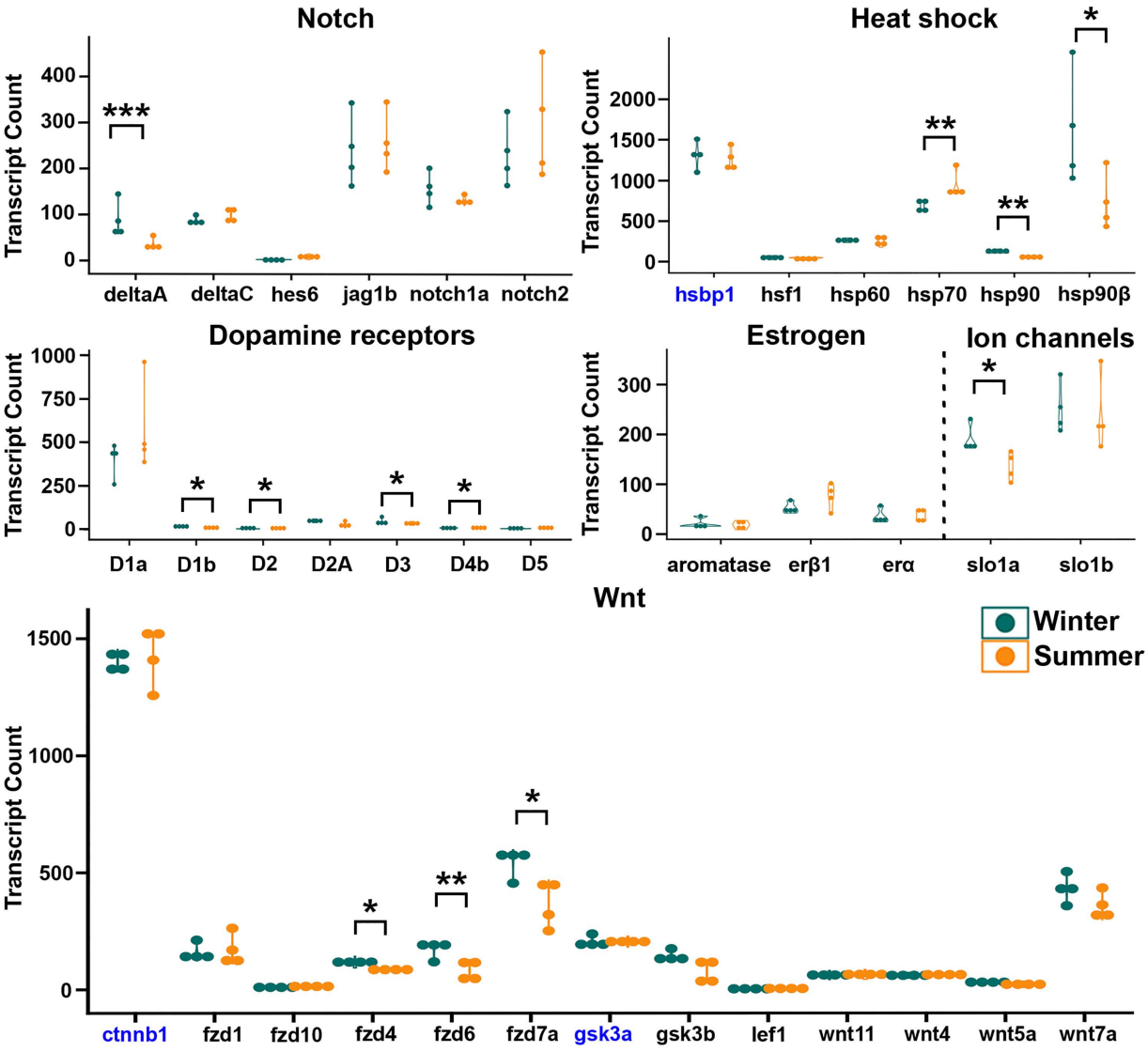

Fig 5. Gene expression in the saccule is lower in the summer for many genes. Expression of 12 of 37 genes was significantly different between seasons. Expression of *hsp70* was significantly upregulated in the summer compared to winter. Expression of *deltaA*, *hsp90*, *hsp90β*, *slo1a*, *fzd4*, *fzd6*, and *fzd7a* were significantly downregulated in the summer compared to winter. Expression of several dopamine receptors was also downregulated in the summer (*D1b, D2, D3, D4b*), although overall transcript numbers were very low for these receptors. *hsbp1*, *ctnnb1*, and *gsk3a* were used as normalization genes (blue). Dots represent individual (pooled) samples, *p < 0.05, **p < 0.01, ***p < 0.001.

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females (normalized transcript counts 5.7–301.2). For example, the D3 receptor was expressed in a moderate range in non-reproductive animals (20.6–53.2), while in reproductive animals the normalized transcript counts spanned a larger range (29.7–150.6). Estrogen pathway genes similarly exhibited wider variability in reproductive lagenae, yet none differ significantly between breeding states. For example, *erα* expression ranged from 31.4–115.4 in non-reproductive females and 29.7–225.9 in reproductive females. Only three genes exhibited seasonal differences: the Wnt receptors *fzd1* and *fzd6,* and the BK channel gene *slo1a*, all of which were expressed at higher levels in non-reproductive lagenae (Fig 4).

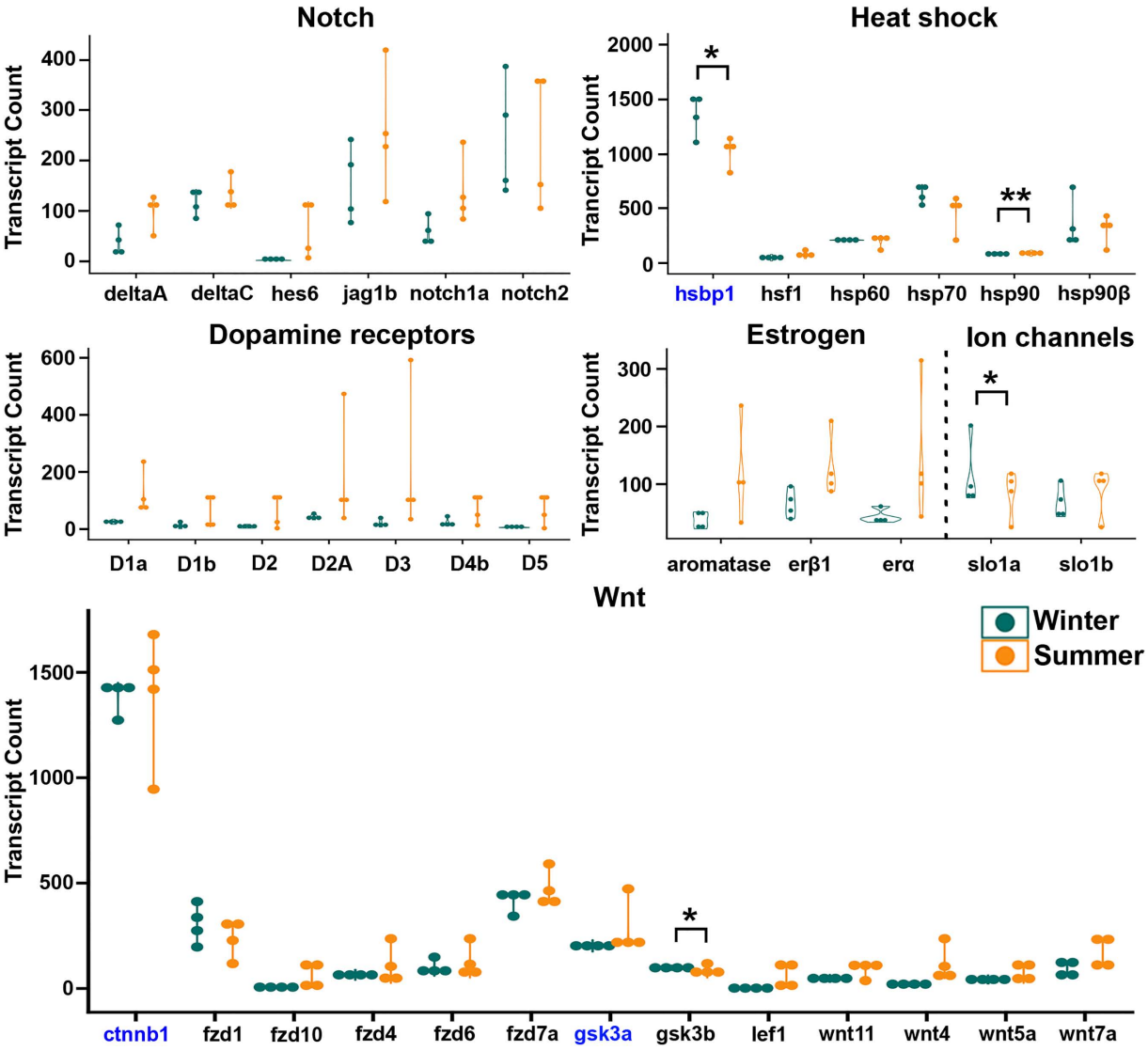

Fig 6. Utricular gene expression is relatively stable between seasons. Expression of four of 37 genes differed seasonally in utricle. *slo1a, hspb1,* and *gsk3b* were significantly downregulated in the summer compared to winter, while *hsp90* was slightly but significantly upregulated in summer. *hsbp1*, *ctnnb1*, and *gsk3a* were used as normalization genes (blue). Dots represent individual (pooled) samples, *p < 0.05, **p < 0.01.

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The largest shift occurred in *fzd6*, which was expressed 2.5-fold higher in non-reproductive fish (mean = 128.5 ± 50.5 sd) relative to reproductive fish (55.5 ± 26.1).

The saccule is the only inner ear organ in female midshipman known to exhibit seasonal changes in hair cell density [14]. Accordingly, we hypothesized that saccules from reproductive females would show elevated expression of genes involved in regulating hair cell number. Overall, saccular gene expression differed for 12 of the 37 genes examined, with the majority of differentially-expressed genes seen at higher levels in non-reproductive samples (Fig 5). Dopamine receptor transcripts were significantly different for *D1b, D2, D3*, and *D4b*. However, transcript counts were very low for all four receptors, potentially causing small, biologically-insignificant changes to appear meaningful. For example, raw transcript counts for *D4b* ranged 2–10 in summer and 3–29 in winter. By contrast, *D1a* was highly expressed (transcript

counts > 250) regardless of reproductive state (Fig 5). Estrogen pathway genes were also consistently expressed at low levels in the saccule and did not differ between seasons (normalized gene counts 24.3–102.0 across seasons). Among the two BK channel genes, *slo1a* was significantly elevated in non-reproductive saccules (190.0 ± 27.6 in winter vs. 135.9 ± 28.7 in summer) (Fig 5).

Several genes associated with hair cell addition or survival showed reproductive-state differences in saccular expression (Fig 5). *Hsp70* expression was 1.4-fold higher in summer saccules, whereas *hsp90* and *hsp90β* were significantly elevated in winter (2.0X and 2.2X higher, respectively). The Notch receptors *notch1a* and *notch2* and the Notch ligand *jag1b* were robustly expressed (gene counts > 100) but did not vary seasonally. In contrast, the Notch receptor *deltaA* showed moderate expression levels and was significantly upregulated in non-reproductive saccules (89.1 ± 38.8 in winter vs. 35.3 ± 13.3 in summer) (Fig 5). The Wnt ligand *wnt7a* and the frizzled receptors *fzd4*, *fzd6*, and *fzd7a* were also highly expressed in saccules across breeding states. For example, normalized transcript counts for *wnt7a* were 297.8–503.3 across breeding states, with no seasonal difference. However, three frizzled receptors, *fzd4*, *fzd6*, and *fzd7a,* were significantly upregulated in non-reproductive saccules, with 1.3-fold, 2.1-fold, and 1.5-fold higher winter expression, respectively (Fig 5).

Although the utricle does not undergo seasonal changes in hair cell density, reproductive females exhibit a significant increase in utricular auditory sensitivity [13,14]. We therefore hypothesized that utricles from breeding-state females would show elevated expression of genes involved in hair cell tuning (*e.g.,* BK channels) or efferent modulation (*e.g.,* dopamine pathway genes), but not genes associated with hair cell turnover. Consistent with this hypothesis, 33 of the 37 genes examined showed stable mRNA expression across breeding states, including all Wnt and Notch pathway genes (Fig 6). Dopamine receptor expression was highly variable in utricles from reproductive females but did not differ significantly between seasons. For example, normalized transcript counts for the D3 receptor were 8.4–40.1 and 35.5–590.9 for utricles from winter and summer fish, respectively. By contrast, the BK channel gene *slo1a* was significantly upregulated in non-reproductive utricles (1.4X higher in winter). Given the established role of this channel in shaping hair cell resonance, this seasonal shift suggests modulation of utricular hair cell tuning across reproductive states (Fig 6).

While not significant, utricles from reproductive females showed a trend toward higher expression of the estrogen receptor genes *erα* and *erβ1*, as well as the estrogen synthesis gene *aromatase* (Fig 6). For these transcripts, the high level of variability may have masked significant trends. For example, normalized transcript counts for *erβ1* in the utricle were 39.9–96.2 in winter and 87.7–209.9 in summer. Unlike the saccule, reproductive-state utricles expressed significantly more *hsp90* and significantly less *hsbp1*, while *hsp70* expression remained unchanged (Fig 6), highlighting organ-specific differences in heat shock pathway regulation. Expression of Wnt signaling genes in the utricle did not significantly differ between seasons, but *wnt7a* expression was lower in the utricle than in the saccule (normalized transcript count across seasons 133.2 ± 66.1 vs. 393.7 ± 66.6 for utricle vs. saccule) (Figs 5, 6).

The saccule has a unique gene expression profile compared to the utricle

We next examined how gene expression varied as a function of both reproductive state and inner ear organ, focusing on the two end organs with known differences in seasonal auditory plasticity. The saccule exhibits seasonal changes in both hair cell density and auditory sensitivity, whereas the utricle shows physiological plasticity without accompanying changes in hair cell density [9,13,14]. Figure 7 shows the log2 fold changes between these organs, with higher values indicating increased expression in the utricle vs. the saccule.

In non-reproductive females, 12 genes were differentially expressed between end organs, with seven expressed at higher levels in the saccule and five more highly expressed in the utricle (Fig 7). Of the seven genes expressed at higher levels in the saccule from winter fish, four (*deltaA, wnt4, wnt7a, notch1a*) represent gene classes important for cell proliferation or hair cell specification. Of the five transcripts more highly expressed in the utricle of winter fish, two (*era, aromatase*) are important for estrogen responses, suggesting that the utricle in winter females may be influenced by estrogen

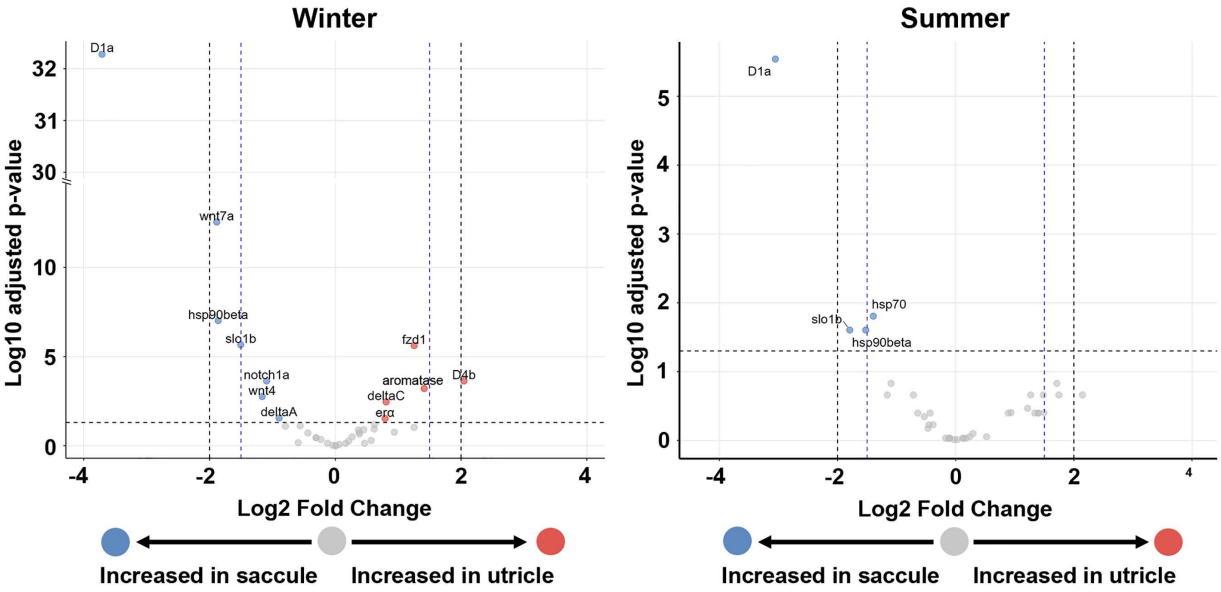

Fig 7. Gene expression changes in the saccule and utricle differ seasonally. Volcano plots showing the log2 fold change differences for normalized transcripts in winter (left) and summer (right). Gene expression is compared such that a fold change of zero represents no difference between the saccule and utricle for a given season. Significantly differentially-expressed genes are indicated by colored dots while gray dots denote genes that do not differ between end organs. Higher values indicate increased expression in the utricle (red dots); lower values show increased expression in the saccule (blue dots). The horizontal dashed line on each graph is the log10 adjusted p-value above which expression differences are considered significant. The vertical dashed lines indicate the 1.5-fold (purple line) and 2-fold (black line) expression differences. Note that the y-axis scale differs between graphs due to the much higher log10 adjusted p-values for some transcripts in the winter sample.

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modulation. By contrast, only four genes were differentially expressed in ears from reproductive females and all showed increase expression in the saccule as compared to the utricle. Interestingly, *slo1b, hsp90ß,* and *D1a* were all highly expressed in the saccule (relative to the utricle) in either season, suggesting that these transcripts may be important for saccular function regardless of reproductive state.

In situ localization reveals cell-specific expression of Notch, Wnt, and Hsp pathway genes

To identify the specific cell types within the sensory epithelia that express genes implicated in seasonal auditory plasticity, we performed hybridization chain reaction (HCR) fluorescent *in situ* hybridization (FISH) on saccules from reproductive (summer) females. Transverse vibratome sections showed that the Notch pathway ligands *deltaA* and *jag1b* were highly expressed in hair cells but were detected in few or no supporting cells (Fig 8 A1-A3 shows *deltaA*, B1-B3 shows *jag1b*).

Within the Wnt pathway, the receptor *fzd7a* was expressed at high levels in hair cells and some supporting cells, whereas the downstream Wnt effector *lef1* was enriched primarily in supporting cell nuclei (Fig 8 C1-D3). This pattern indicates that supporting cells are recipients of at least some Wnt-derived signals, although the cellular source of the ligand (hair cell, supporting cell, or other cell type) remains unresolved.

We also found that both supporting cells and hair cells expressed high levels of transcripts for *hsp70* (Fig 8 E1-E3), consistent with its widespread induction in response to physiological or environmental stressors [32,33]. It is notable that, while mRNAs for *deltaA, jag1b, fzd7a*, and *hsp70* were detected in the cytoplasm, *lef1* mRNA was detected mostly in the nucleus.

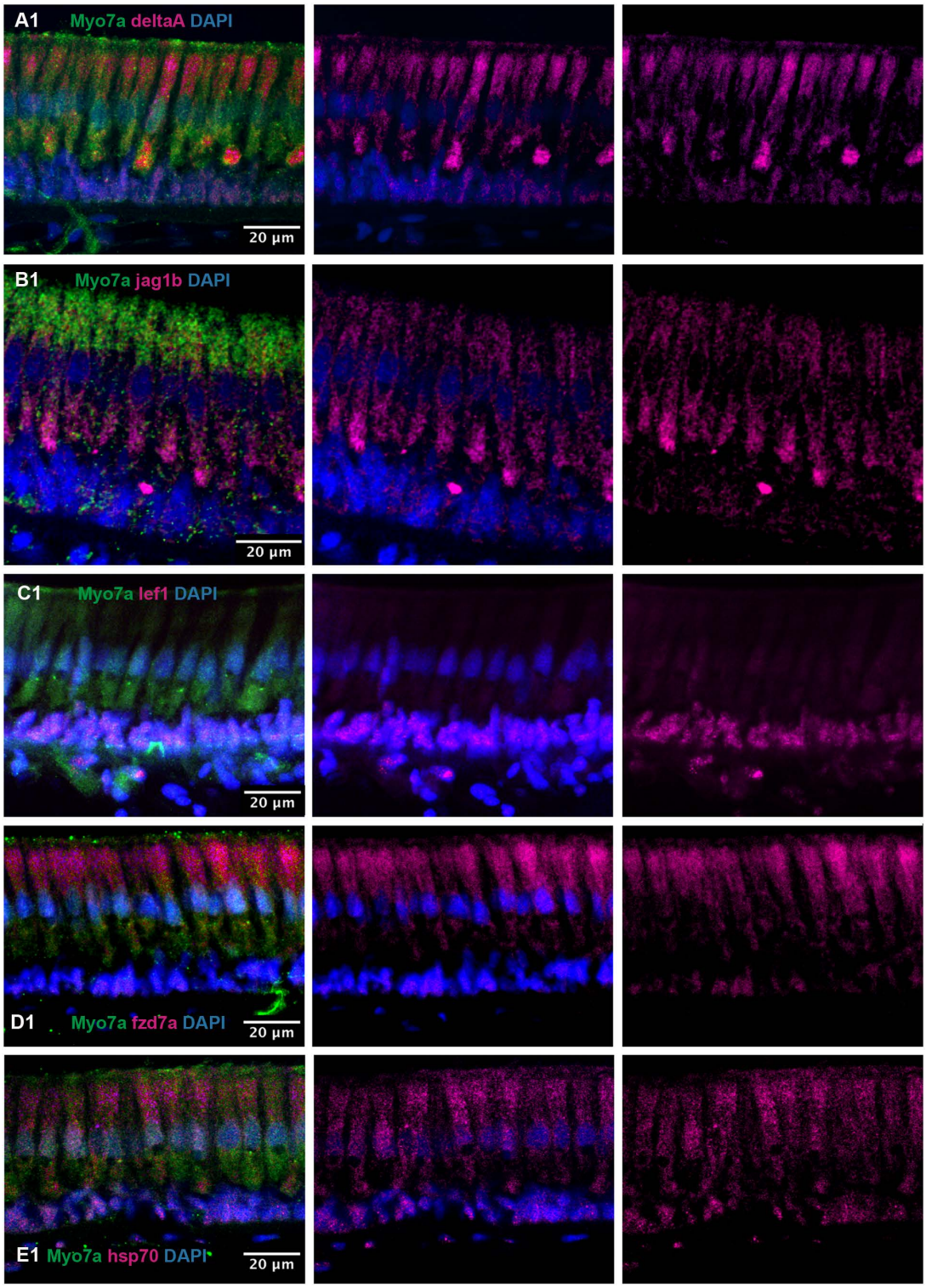

Fig 8. Localization of a set of transcripts for three signaling pathways. All panels show confocal images of transverse sections of saccules from summer midshipman fish that were labeled by probes for each transcript (*magenta*), antibodies to the hair cell (HC) marker Myo7a (*green*), and the nuclear marker DAPI (*blue*). The positions of hair cell nuclei (HC Nu), supporting cell nuclei (SC Nu) and stroma (connective tissue under the sensory

epithelium) are indicated. A1-A3 show labeling for the *deltaA* probe in the same slice/field; all labels are shown in A1; *deltaA* and DAPI are shown in A2; and d*eltaA* only is shown in A3. The arrow points to a hair cell with a high expression level of *deltaA*. Labeling for *jag1b, lef1, fzd7a,* and *hsp70* are shown using a similar formatting in B1-B3, C1-C3, D1-D3, and E1-E3, respectively. Arrows point to highly labeled cells.

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Discussion

Seasonal plasticity of auditory thresholds in the plainfin midshipman fish is likely due to a combination of mechanisms. In the saccule, reduced thresholds (*i.e.,* increased sensitivity) in reproductive females are correlated with increased cell proliferation and a corresponding increase in hair cell density [14,34], suggesting that saccular sensitivity is regulated, at least in part, by an increase in receptor cell number. In contrast, utricular physiological plasticity occurs without corresponding changes in hair cell number, suggesting involvement of alternative mechanisms such as changes in hair cell tuning and/or efferent modulation. To evaluate these possibilities, we examined expression of genes associated with cell addition/survival as well as genes that likely modulate hair cell activity, including BK channels and dopamine receptors.

We show that inner ear mRNA profiles share common patterns not seen in a non-auditory tissue (gill). We further show that the saccule, which experiences seasonal changes in both morphology and physiology, has a distinct gene expression profile compared to the other inner ear organs (lagena and utricle), which do not show seasonal plasticity in hair cell number. Both the lagena and utricle showed highly stable transcript levels across reproductive states: 33–34 of 37 genes assayed were unchanged between seasons, although the small subset of seasonally responsive transcripts differed between the two end organs. By contrast, 32.4% (12 of 37) of the transcripts changed seasonally in the saccule, consistent with our hypothesis that the saccule would show more dynamic changes in gene expression, correlated with the increased cell proliferation and hair cell addition observed in the saccule from reproductive females.

Genes associated with cell proliferation and hair cell addition

We examined gene expression in the Wnt and Notch signaling pathways because they play well-recognized roles in inner ear development and hair cell regeneration across vertebrates [19,20,35–38]. Wnt proteins are secreted ligands that bind to transmembrane receptors (frizzled) on target cells, activating intracellular ß-catenin, which in turn activates the TCF/LEF transcription factors, upregulating Wnt target genes [39,40]. Studies in mouse and chick inner ear demonstrate the necessity of canonical Wnt signaling for cell proliferation during cochlear development [41–44]. Genetic or pharmacologic manipulation of Wnt signaling also alters progenitor cell proliferation and hair cell regeneration in the zebrafish lateral line, demonstrating the evolutionarily conserved role for Wnt in hair cell production [19,20,45–47]. Expression of both Wnt signaling genes and estrogen receptors is altered during regeneration in the chick inner ear [48], and estrogen disrupters reduce Wnt signaling and progenitor cell proliferation in the developing brain [49], suggesting that reproductive hormones may modulate Wnt signaling in seasonally plastic tissues.

Given the increased cell proliferation and hair cell density in the saccule of reproductive-state midshipman females [14,34], we hypothesized that Wnt signaling genes would show higher expression in fish collected in the summer reproductive season. However, of the 13 Wnt pathway genes that we examined, 10 maintained stable expression levels across seasons within the saccule, including all four secreted ligands (*wnt4, wnt5a, wnt7a,* and wnt*11*) and the Wnt effector *lef1*. The only significant difference in Wnt pathway expression was reduced expression of several frizzled receptors (*fzd4, fzd6,* and *fzd7a*) in saccules from reproductive females. We observed a similar pattern in the lagena, a tissue where we did not expect seasonal changes in Wnt gene expression; both *fzd1* and *fzd6* transcript levels decreased in lagenae from summer females. By contrast, gene expression levels for Wnt ligands and receptors did not change seasonally in the utricle, consistent with our hypothesis of a lack of seasonal hair cell proliferation. Our *in situ* hybridization experiment confirmed that the *fzd7a* receptor was expressed in saccular hair cells and supporting cells, while the Wnt effector *lef1* was primarily expressed in supporting cells. Lef1 regulates cell proliferation in many tissues, including neuronal precursors,

and a *lef1* mutation reduces cell proliferation in the developing zebrafish lateral line [45,50,51]. Therefore, it is feasible that Wnt signaling may mediate cell proliferation in the midshipman saccule.

It is unclear why the genes we selected did not change in the manner we predicted (*i.e.,* increased Wnt signaling in saccules from summer females). As we did not query expression of every Wnt gene, we may have missed key members of the Wnt pathway responsible for seasonal plasticity in the saccule. It is also possible that the tissue sources of Wnts do not reside within the sensory organs themselves; we did not sample surrounding tissues. Another interpretation is that Wnt activity does indeed increase in reproductive females but it is not correlated with transcriptional changes. Furthermore, we may have failed to catch the period of increased Wnt expression, which we discuss further below. Finally, Wnt may not be a key regulator of seasonal hair cell addition in this species. Further studies are needed to better understand if Wnt may control hair cell numbers across seasons in the female midshipman saccule.

While Wnt signaling is a major regulator of cell proliferation, Notch signaling plays a critical role in regulating hair cell differentiation via lateral inhibition [21,36,52–54]. For example, in the embryonic mouse cochlea, undifferentiated sensory cell precursors expressing the Notch ligand Jag2 activate Notch in neighboring cells, preventing the upregulation of the pro-hair cell transcription factor, *Atoh1*, thus laterally inhibiting these cells from acquiring the hair cell fate [55]. Genetic or pharmacologic Notch inhibition results in increased hair cell production during development and regeneration following acute toxic injury [22,36,56,57]. For example, *Jag2* loss-of-function mutation leads to hair cell overproduction in the mouse cochlea [57]. Similarly, mutations in *mib*, an E3 ligase that regulates Notch pathway activity, cause increased hair cells at the expense of supporting cells in the zebrafish mind-bomb mutant [58–60]. Notch signaling also modulates hair cell regeneration, with Notch inhibition causing increased hair cell production in the zebrafish lateral line and avian and mammalian inner ears [22,56,61,62]. Further, estrogen can decrease Notch signaling, suggesting that seasonal changes in estrogen could regulate hair cell differentiation in the midshipman inner ear [63,64].

The Notch receptors *deltaA* and *jag1b* were expressed in hair cells in saccules from reproductive females, with high expression levels of *deltaA* in a subset of cells. Prior studies show that *Delta1* (in chick) and *deltaA* (in zebrafish) are highly upregulated in dividing precursors and newly differentiating hair cells during regeneration [22,53]. Therefore, this population of high *deltaA*-expressing cells may represent recently generated hair cells, consistent with our previous finding of immature-appearing hair cells in the saccule of reproductive female midshipman [14]. Surprisingly, only one Notch pathway gene, *deltaA,* differed seasonally in saccules, showing reduced expression in reproductive females. In prior studies of damaged chicken sensory organs, we detected a significant increase in *Delta1* transcripts in sensory epithelia that primarily localized to the newly formed hair cells [61,65]. This expression pattern is consistent with the regenerating hair cells' inhibition of supporting cells from transdifferentiating to hair cells, via Delta1-Notch signaling. Here, we saw fewer *deltaA*-expressing cells in summer than in winter, which could indicate that the period of hair cell addition was significantly curtailed by the time we examined gene expression. No seasonal change in Notch pathway gene expression was seen in the lagena or utricle, consistent with our observations of stable hair cell numbers in these epithelia across seasons [14].

Our probe set was based on sequences obtained from our prior study using bulk RNA-Seq of the whole midshipman fish inner ear and did not capture the full suite of Notch signaling genes [17]. Therefore, it is possible that we missed some Notch pathway members that may be important for seasonal changes in hair cell differentiation in the midshipman fish saccule. As mentioned above, mutations in *Jag2* in mice and *mib* in zebrafish cause an increase in hair cells at the expense of supporting cells [57,58], and neither of these genes was present in our probe set. Further, many mammalian genes have two paralogs (also referred to as onologues) in fishes due to a whole genome duplication event during early evolution of teleost fish [66–68]. Unfortunately, our probe set did not target both paralogs (*e.g.,* we examined *notch1a* but not *notch1b*), again due to limitations stemming from the lack of a fully annotated midshipman genome.

Finally, it remains possible that neither Notch nor Wnt pathways are responsible for the reproductive increase in saccular hair cells. Other cellular pathways such as fibroblast growth factor (FGF) signaling could be a primary driving force for seasonal increases in hair cell addition. FGF signaling plays a critical role in inner ear development and hair cell

regeneration, likely by modulating both cell proliferation and cell differentiation [18,69–73]. Estrogen can regulate the proliferative effect of FGF-2 in neuronal progenitor cells and enhances tumor expansion in a breast cancer cell line through an FGF-mediated mechanism [74,75]. Therefore, FGF signaling is another target of interest to regulate seasonal plasticity in cell proliferation in the midshipman saccule. Future studies using single cell RNA-Seq are necessary to resolve the full suite of transcriptional changes correlated with cell proliferation and hair cell addition in the midshipman saccule.

Genes associated with cell survival

Heat shock proteins (Hsps) play critical roles in neuronal survival following injury and may be targets to ameliorate Parkinson's Disease and other neurodegenerative diseases [76–79]. In the mouse utricle, *Hsp70* is upregulated in supporting cells during ototoxic hair cell damage and *Hsp70* over-expression significantly attenuates hair cell death, suggesting that *Hsp70* plays a critical role in mitigating acute hair cell toxicity [23,80–83]. Hsps act as molecular chaperones to facilitate protein folding and mediate survival in response to cellular stress [84,85]. Although *hsp* expression is rapidly upregulated by cellular stress, *Hsp70* is also expressed in the guinea pig cochlea under baseline conditions, demonstrating that acute damage is not required to initiate a heat shock response [86].

We initially hypothesized that Hsp genes would be upregulated in the saccules of reproductive females, enhancing hair cell survival and contributing to increased hair cell density by maintaining existing cell populations. In contrast, we did not expect seasonal changes in Hsp signaling genes in the lagena or utricle, given the absence of seasonal plasticity in hair cell number in those epithelia. Contrary to our initial prediction, expression of *hsp70*, *hsp90*, and *hsp90β* was deceased in saccules from reproductive females relative to the non-reproductive condition. These results are consistent with our recent finding that cell proliferation increases in the saccule of reproductive females without a corresponding change in cell death [34]. Importantly, *in situ* hybridization confirmed *hsp70* expression in both hair cells and supporting cells in saccules from summer females, lending confidence to our nCounter results that *Hsps* are indeed expressed in the midshipman fish ear.

It is unclear why some Hsp-related genes were downregulated in the saccule of reproductive females. In the winter, fish are collected via otter trawl and brought to the surface from a depth of 60 m or more, a process that likely induces substantial acute cellular stress. However, because non-reproductive fish were held under laboratory conditions for two weeks before tissue collection, collection-related stress should have been largely mitigated. Further, *hsp* expression was stable across seasons in the gills, indicating that neither collection nor housing conditions differentially activated systemic hsp signaling. Collectively, our data suggest that heat shock signaling does not contribute to seasonal increases in auditory sensitivity in reproductive female midshipman.

Modulation of hair cell activity

One unexpected result was that the K^+ ion channel transcript *slo1a*, a potential contributor to auditory physiological plasticity, was significantly reduced in all three organs of summer reproductive females (Figs 4–6). Hair cell tuning is regulated in part by BK channel expression and kinetics [87,88]. Rohmann et al. [16] found that reproductive midshipman fish had increased *slo1a* and *slo1b* expression in the inner ear and that pharmacological inhibition of BK channels increased saccular thresholds in reproductive males, bringing them close to levels observed in non-reproductive animals. While both *slo1a* and *slo1b* were upregulated in reproductive animals, only *slo1b* expression was significantly correlated with threshold changes within individual saccular hair cells of reproductive females [16]. By contrast, we did not detect seasonal changes in *slo1b* expression. In our study, *slo1b* levels were relatively high (>200) in the saccule across seasons, while transcript levels were lower for both the utricle and lagena, again with no seasonal differences.

Seasonal dynamics of circulating steroid hormones may partially explain these discrepancies. In female midshipman fish, estradiol levels are very low in the winter, rise sharply in March, peak in April, and decline to near-winter levels by June [89]. Our summer samples were collected from May-July. If BK channel expression is both estrogen-dependent and highly dynamic, elevated *slo1a*/*1b* expression in early summer could decline by mid-summer, even if the protein persists

in the membrane. We pooled samples from multiple summer collecting sessions, which may have masked our ability to detect rapid changes in transcript levels within the summer breeding season.

Although BK channels can shape hair cell resonance and frequency tuning, dopaminergic efferents may also modulate hair cell excitability in the midshipman inner ear [4,15]. Dopamine application increases saccular thresholds in reproductive females to levels comparable to non-reproductive fish, and this effect is abolished by a D2 antagonist, indicating that dopamine acts via an inhibitory D2-mediated mechanism to reduce hair cell sensitivity during the non-reproductive season [15]. Perelmuter et al. [15] reported decreased *D2a* expression in the summer saccule, with no seasonal changes in other *D1*/ *D2* receptor subtypes.

We found significant seasonal differences in the saccule for several dopamine receptors; D1b, D2, D3, and D4b were all expressed at higher levels in the saccule of non-reproductive females compared to the reproductive condition. However, these differences were only seen in receptors with low raw transcript counts. Low counts reduce the signal to noise ratio by artificially inflating small differences, which can bias the analysis [90,91]. Therefore, we think it unlikely that these differences are biologically meaningful. By contrast, *D1a* was expressed at relatively high levels (>200) across seasons, suggesting that excitatory dopamine signaling may modulate saccular activity regardless of season. Dopamine receptor levels were more variable in the lagena and utricle in our study but did not change seasonally. These patterns suggest that dopamine may modulate hair cell sensitivity differently across end organs within the same animal. Future pharmacological studies will be important for determining how dopaminergic signaling affects auditory thresholds in the utricle and lagena and for clarifying the extent to which dopamine contributes to seasonal modulation of auditory sensitivity.

Estrogen pathway gene expression

Circulating estrogen levels are correlated with saccular auditory thresholds in female midshipman and estrogen implants in non-reproductive females phenocopy the reproductive auditory phenotype, demonstrating that estrogen is a key driver of auditory plasticity in this species [9,92]. We therefore expected to see increased expression of estrogen signaling components in the saccule and utricle of reproductive females, concurrent with estrogen-mediated physiological changes in these end organs. Unexpectedly, we did not observe seasonal differences in *aromatase*, *era,* or *erb1* expression in any inner ear end organ. Although there was a trend towards high estrogen pathway expression in the utricle of summer females, substantial variability may have obscured statistically significant differences. We did find higher transcript levels of *aromatase* and *era* in the utricle of winter females as compared to the saccule, suggesting heightened estrogen responsiveness in the utricle during the non-reproductive season, perhaps in preparation for breeding. Importantly, e*ra* was significantly upregulated in gills of non-reproductive females, demonstrating that our assay reliably detects differential expression and supporting the validity of our inner ear findings.

While prior research demonstrates expression of *era, erb,* and *aromatase* in the female midshipman inner ear [17,93], we are not aware of studies reporting quantitative seasonal differences in estrogen receptor expression in midshipman. This absence of receptor-level plasticity suggests that fluctuating circulating hormone concentrations, rather than local changes in receptor abundance, may be the primary drivers of seasonal auditory modulation. Future experiments are required to test this hypothesis.

Conclusions and limitations

We show correlations between gene expression and seasonal auditory plasticity, with greater seasonal changes in transcript levels in the saccule as compared to other inner ear end organs. However, our data did not support our hypothesis that Wnt and Notch signaling would increase in the saccules of reproductive females, as we predicted based on the activating roles of these pathways upon cell proliferation and hair cell addition. Several limitations to our study warrant consideration, as we discuss below.

First, although there is strong evidence for a seasonal increase in saccular hair cell number in reproductive female midshipman, the precise time course of this increase, from the winter non-reproductive period through the reproductive pre-nesting and nesting phases, is unknown and likely varies annually based on water temperature and weather patterns (*e.g.,* El Niño) [14,94] (and our unpublished observations). Female midshipman experience a pronounced spike in circulating steroid hormones during the pre-nesting period (April and May), followed by a sharp decrease to near-winter levels during active nesting in the breeding season (June and July) [89]. Our reproductive-state animals were collected in May-July, spanning the period of estrogen decline. Likewise, although our non-reproductive females were collected in January, prior to the spring spike in estrogen levels, some females had GSI values ~ 4, indicating early gonadal recrudescence. Thus, some "winter" fish may have been transitioning into the pre-nesting condition, while some "summer" fish collected later in the season may represent post-nesting individuals. This hypothesis is supported by our analysis of differentially expressed genes between the saccule and utricle, where we saw significant increases in multiple Wnt and Notch genes in the saccules, but not the utricles, of winter females (Fig 7), consistent with our hypothesis that Wnt and Notch may regulate cell proliferation and hair cell maturation in the saccule from pre-nesting females.

Second, despite our efforts to randomly distribute RNA across samples for a given season, variability in fish weight or GSI between pooled samples may have influenced our results. In the summer samples, sample 3 fish were larger and had higher GSI than the other three samples, which could have skewed the results. Based on the heat maps in Fig 2, summer sample 3 did not appear to vary substantially in expression pattern for the saccule or lagena, although this sample may have carried additional weight for gill and utricle tissue results. We consider this unlikely, given that we found few transcripts that differed seasonally within gill or utricle tissues. However, this sampling structure likely contributed to the high variability we observed for certain transcripts and may have masked additional trends in our data. Future studies should characterize inner ear gene expression during the early pre-nesting period and explicitly correlate transcript levels with circulating estrogen concentrations at the level of the individual animal.

Another limitation relates to our tissue collection technique, in that we opted to perform rapid dissections to minimize the chance of mRNA degradation. Therefore, there were non-epithelial tissues present in our samples, including loose connective tissues and glial cells, which may have obscured changes in gene expression in the sensory epithelia themselves. While non-sensory expression is unlikely for BK channels, which are known regulators of hair cell tuning [16,87,88,95], it remains a possibility for the other gene families examined here. Importantly, our HCR analyses demonstrate that several key Wnt and Notch transcripts localize to hair cells and/or supporting cells, increasing confidence that our nCounter results likely represent transcript levels in the sensory epithelium.

Finally, decisions in the data analysis pipeline may have influenced our findings. The saccule in midshipman is substantially larger than the utricle or lagena, yet we did not normalize transcript counts to cell number within each end organ. However, we did not see marked differences in transcript counts across tissues, suggesting that cell number was unlikely to be a major confound. Additionally, we used a highly conservative approach to normalize our data. The transcript counts that we obtained for saccules had relatively low variation when compared to the lagenae and utricles. Because our goal was to compare expression across all three inner ear organs, we selected reference genes using the geNorm algorithm applied to all tissues, rather than identifying organ-specific normalization genes. Although this approach may reduce sensitivity to organ-specific changes, it provides a stringent and uniform standard for cross-organ comparisons.

Despite these limitations, our findings provide new insights into gene expression in the fish sensory organs of the inner ear and provide a framework for future studies to determine the different mechanisms by which seasonal, hormonally-driven auditory plasticity is established within the utricle and saccule. A deeper understanding of these processes in midshipman fish is likely to inform mechanistic models of seasonal modulation of hearing in other vertebrate taxa.

Supporting information

S1 Table. Genes names and probe sequences for the 37 target genes. Gene names in blue represent housekeeping genes.
(PDF)

S1 File. nCounter gene expression data. Data are presented as normalized transcript counts for each tissue sample (four winter and four summer samples), accompanied by the Log2 fold change, linear fold change, and p-values for each transcript in each tissue. Positive values for Log2fold change data indicate transcripts that are more highly expressed in winter relative to summer, while linear fold changes are only represented as positive values (because all transcript counts are above zero). Transcripts differentially expressed between seasons with a tissue are highlighted with red text. Subsequent tabs on the associated file show each tissue separately. Note that the same fish were used for all tissues such that total length, gonad mass, fish mass, and GSI values are the same on all tabs.
(XLSX)

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Author contributions

Conceptualization: Coty Jasper, Jennifer S. Stone, Joseph A. Sisneros, Allison Coffin.

Data curation: Coty Jasper, Robin Gibson, Allison Coffin.

Formal analysis: Coty Jasper, Robin Gibson, Jennifer S. Stone, Allison Coffin.

Funding acquisition: Jennifer S. Stone, Joseph A. Sisneros, Allison Coffin.

Investigation: Coty Jasper, Sumaya Hassan, Robin Gibson, Tot Nguyen, Olivia Molano, Leila Farbod, Jennifer S. Stone, Joseph A. Sisneros, Allison Coffin.

Methodology: Coty Jasper, Sumaya Hassan, Robin Gibson, Tot Nguyen, Olivia Molano, Leila Farbod, Jennifer S. Stone, Allison Coffin.

Project administration: Jennifer S. Stone, Joseph A. Sisneros, Allison Coffin.

Resources: Jennifer S. Stone, Joseph A. Sisneros.

Supervision: Robin Gibson, Jennifer S. Stone, Joseph A. Sisneros, Allison Coffin.

Validation: Coty Jasper, Tot Nguyen, Olivia Molano, Leila Farbod, Jennifer S. Stone, Allison Coffin.

Visualization: Coty Jasper, Sumaya Hassan, Robin Gibson, Tot Nguyen, Jennifer S. Stone, Allison Coffin.

Writing – original draft: Coty Jasper, Robin Gibson, Jennifer S. Stone, Joseph A. Sisneros, Allison Coffin.

Writing – review & editing: Coty Jasper, Sumaya Hassan, Robin Gibson, Tot Nguyen, Olivia Molano, Leila Farbod, Jennifer S. Stone, Joseph A. Sisneros, Allison Coffin.

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