Brain, Behavior and Evolution

Research Article

Brain Behav Evol
DOI: 10.1159/000543759

Received: September 3, 2024
Accepted: January 20, 2025
Published online: January 30, 2025

Brain Activation Patterns and Dopaminergic Neuron Activity in Response to Conspecific Advertisement Calls in Reproductive versus Non-Reproductive Male Plainfin Midshipman Fish (*Porichthys notatus*)

Brooke J. Vetter[a] Jonathan T. Perelmuter[b] Nicholas R. Lozier[c]
Joseph A. Sisneros[d, e, f] Paul M. Forlano[g, h, i, j]

[a]Department of Biology, University of St. Thomas, St. Paul, MN, USA; [b]Department of Neurobiology and Behavior, Cornell University, Ithaca, NY, USA; [c]Department of Neurobiology, University of Pittsburgh, Pittsburgh, PA, USA; [d]Department of Psychology, University of Washington, Seattle, WA, USA; [e]Department of Biology, University of Washington, Seattle, WA, USA; [f]Virginia Merrill Bloedel Hearing Research Center, Seattle, WA, USA; [g]Department of Biology, Brooklyn College, City University of New York, Brooklyn, NY, USA; [h]Program in Ecology, Evolution, and Behavior, The Graduate Center, City University of New York, New York, NY, USA; [i]Program in Neuroscience, The Graduate Center, City University of New York, New York, NY, USA; [j]Program in Behavioral and Cognitive Neuroscience, The Graduate Center, City University of New York, New York, NY, USA

Keywords
Acoustic communication · Auditory pathways · Dopamine · Fish · Teleost

Abstract
Introduction: The plainfin midshipman fish (*Porichthys notatus*) relies on the production and reception of social acoustic signals for reproductive success. During spawning, male midshipman fish produce long duration advertisement calls to attract females, which use their auditory sense to locate and access calling males. While seasonal changes based on reproductive state in inner-ear auditory sensitivity and frequency encoding in midshipman are well documented, little is known about reproductive-state-dependent changes in central auditory sensitivity and auditory neural responsiveness to conspecific advertisement calls. Previous research indicates that forebrain dopaminergic neurons are preferentially active in response to conspecific advertisement calls and during female auditory-driven behavior in the breeding season. These dopamine neurons project to both the inner ear and central auditory nuclei and contribute to regulation of inner-ear auditory sensitivity based on reproductive state. The present study tested the hypothesis that exposure to the male advertisement call would elicit differential activation in auditory brain nuclei and in the forebrain auditory-projecting dopaminergic nucleus in reproductive versus non-reproductive male midshipman. ***Methods:*** Fish were collected during the spring reproductive and winter non-reproductive months and were exposed to a playback of the advertisement call or ambient noise (control). Immunohistochemistry identified activated neurons (pS6-ir; proxy for neural activation) in midbrain and forebrain auditory and dopaminergic nuclei. ***Results and Conclusions:*** Our results revealed that in key auditory

karger@karger.com
www.karger.com/bbe

Correspondence to:
Brooke J. Vetter, brooke.vetter@stthomas.edu

Karger

and dopaminergic areas, the greatest activation (most pS6-ir cells) occurred in reproductive males exposed to the advertisement call.

Introduction

Acoustic communication is a key aspect of social behavior across vertebrate groups. Vocalizations are utilized in a variety of social behaviors, including aggressive interactions and reproduction, and an individual's ability to detect and discriminate between these socially relevant auditory signals above the background noise can be essential for survival and reproduction. The neural pathways responsible for detecting and responding to acoustic cues are posited to be conserved across vertebrate taxa [1–4] and vocal-acoustic communication is believed to have evolved first in bony fishes (Osteichthyes) [5, 6]. Understanding the neural circuitry involved in detecting socially relevant acoustic cues in bony fishes could provide valuable insights into the conserved and convergent auditory and vocal-acoustic communication networks across vertebrates.

The plainfin midshipman fish (*Porichthys notatus*) offers an ideal study system for investigating the neural substrates essential for attention and processing of socially relevant acoustic stimuli as their reproductive success depends on vocal-acoustic communication [5, 6]. This species exhibits three adult sexual phenotypes (females and type I and II males) with well-characterized reproductive strategies [7–10]. Type I or nest guarding males produce long duration vocalizations ("hums") to attract gravid (reproductive) females to their nest. For a successful reproductive cycle, females localize the type I male's advertisement call, enter the nest, and deposit eggs, which are promptly fertilized by the male. Type II males "steal" fertilizations from the nest guarding male by entering the nest concurrently with a female and depositing sperm [9]. After fertilization, type I males guard the nest and care for larvae [8]. Nesting type I males will continue to produce advertisement calls to facilitate multiple spawning events throughout the reproductive season [7, 9, 10]. Since multiple type I males establish nesting sites in close proximity, calling males may detect and respond to nearby conspecific competitors [5], especially since spectral aspects of the advertisement call (e.g., amplitude and fundamental frequency) correlate with information about male body size and condition [11].

Male and female midshipman fish undergo seasonal changes in hearing sensitivity related to their reproductive state. It is well documented that during the reproductive season (spring/summer), male (type I and II) and female midshipman fish exhibit decreased hearing thresholds (increased sensitivity) compared to the non-reproductive (NR) season (winter) [12–18]. However, much of this research has focused on the plasticity of the midshipman peripheral auditory system, with limited studies examining reproductive-state-dependent changes in the central auditory system in plainfin midshipman [14 and see 19 for a review].

The auditory pathways in plainfin midshipman have been well characterized using tract tracing techniques and single-unit recordings. In this species, the saccule is one of the primary peripheral sensory structures for hearing [12, 20–22]. Saccular afferent neurons project to nuclei in the auditory hindbrain, which in turn project to the nucleus centralis of the torus semicircularis (TS) (TSnc) in the midbrain [23–25]. The TSnc then projects to the compact division of the central posterior nucleus of the thalamus (CPc), which has reciprocal connections with the hypothalamic anterior tuberal nucleus (AT) [24, 25].

The periventricular posterior tuberculum (TPp) in ray-finned fish is a nucleus in the diencephalon populated with dopaminergic neurons [26 and see 27–29 for reviews]. Tyrosine hydroxylase (TH) is the rate-limiting enzyme for catecholamine synthesis [30] and thus is a marker for both dopaminergic and noradrenergic neurons in the brain. Consistent with studies in other teleost species, TH immunoreactivity (-ir) but absence of dopamine (DA) beta hydroxylase-ir, an enzyme that is required to catalyze DA to norepinephrine, supports the identity of TH-ir TPp neurons as dopaminergic in midshipman fish [31; also see 32 and refs within]. In plainfin midshipman, tract tracing studies have revealed that these dopaminergic neurons in the TPp send direct efferent input to the saccule and the cholinergic octavolateralis efferent nucleus [32, 33]. Additionally, the TH-ir neurons of the TPp send ascending projections to the CPc and areas of the basal forebrain that are thought to be homologous to centers of reward and decision-making across vertebrates [34, 35].

It is well documented that auditory nuclei in the midshipman's midbrain, forebrain, and the dopaminergic TPp respond preferentially to the advertisement hum compared to control or ambient sounds in all three sexual phenotypes (females and type I and II males) [30–33]. The TPp is particularly associated with increased activation in reproductive males (type I and II) and females in response to the male advertisement call [36–39]. Additionally, in female midshipman, there are differences in TPp neuron area based on reproductive state, with reproductive females exhibiting increased TPp neuron area compared with NR females [14].

We hypothesized that the neurons in three higher level auditory nuclei (TSnc, CPc, AT) and the dopaminergic neurons (DAergic) of the TPp in type I males would be differentially activated when exposed to conspecific advertisement calls based on their reproductive state (reproductive vs. NR) and acoustic stimuli (male advertisement call vs. ambient sound control). To assess neural activation in well-defined auditory nuclei in midshipman [24], we used immunohistochemistry (IHC) methods to label for phospho-S6 ribosomal protein (pS6), a marker for phosphorylated ribosomal proteins and an indicator of increased protein translation in response to a stimulus or behavioral state in combination with an antibody against the Hu protein, which specifically labels mature neuronal somata and nicely delineates the cytoarchitecture of the brain [40–42]. Phospho-S6 ribosomal protein is becoming a common marker of neural activation in vertebrates, including fish, due to its robust signal and high specificity [43–50 and see 51–53 for reviews]. In order to quantify activation of DAergic neurons in the TPp, we double labeled for TH-ir and pS6-ir (see above). We predicted that type I males exposed to a conspecific male advertisement call would show greater pS6 activation in the auditory (TSnc, CPc, AT) and DAergic (TPp) neurons compared to the control but only in the reproductive state.

Methods

Fish Collection and Husbandry

NR type I male midshipman fish were collected by otter trawl in January (winter) in Monterey Bay near Moss Landing, CA (R/V John H. Martin, Moss Landing Marine Laboratories), at depths from 85 to 100 m. Reproductive type I male midshipman fish were hand-collected during morning low tides in May (spring) from the same nesting sites in Tomales Bay near Marshall, CA, as in previous studies [e.g., 14, 37, 38]. After collection, fish were transported in coolers with aerated sea water to the University of California Bodega Marine Laboratory (BML) in Bodega Bay, CA, where they were housed in flow-through sea water aquaria at natural ambient temperatures (13–14.5°C) until playback trials, which were conducted within 16–48 h after collection. This holding time is consistent with similar studies on type I [38] and type II [37] male midshipman, as well as female midshipman [36]. Furthermore, Sisneros and Bass [12] reported no decrease in auditory sensitivity to frequencies contained within the male mate call up to 25 days post-collection.

Playback Trials and Data Collection

Playback trials were performed at BML in a large cylindrical outdoor concrete tank (4 m diameter, 0.5 m water depth) during nighttime sessions. A speaker (Aquasonic AQ339, Clark Synthesis, Littleton, CO, USA) was suspended at the center of the tank, positioned 5 cm above the tank floor, and powered by an audio amplifier (BG-1120, TOA Electronics, Secaucus, NJ, USA). A mesh playback cage (240 cm diameter) was placed around the speaker. At the start of each trial, fish were placed in a plastic mesh cylindrical "release cage" (30 cm diameter) situated in a fixed location along the wall of the playback cage (Fig. 1). The sound pressure level (SPL) at the release cage was 130 dB re: 1 μPa, which is consistent with SPLs recorded from the nest entrance in calling type I males [54] and used in previous studies [36–39]. As fish were allowed to swim freely, the mesh playback cage ensured that the fish were exposed to at least 130 dB re: 1 μPa SPL during playback of the male advertisement call. SPLs were calibrated nightly using a mini-hydrophone (model 8103, Bruel & Kjaer, Naerum, Denmark), a charge amplifier (Bruel & Kjaer model 2692), and an oscilloscope (TBS 1064, Textronix, Beaverton, OR, USA).

Male midshipman fish were exposed to either 15 min of continuous male advertisement calls ("hum"; NR: $N = 5$; reproductive: $N = 6$; Fig. 2) or a no-sound ambient control (105–120 dB re: 1 μPa; NR: $N = 6$; reproductive: $N = 7$). The hums were recorded from reproductive type I midshipman collected from nests in Brinnon, WA, during low tides in June/July and housed at the Friday Harbor Marine Laboratory (FHL) in outdoor tanks outfitted with artificial nests. Brantley and Bass [9] showed that the fundamental frequency of male advertisement calls increases with temperature, and female preference reflects this correlation [20]. Therefore, the water temperature at BML was taken nightly (January: 13°C; May 14–14.5°C), and we used FHL recordings taken at the corresponding temperatures to create 15-min audio files from a single calling male with fundamental frequencies of 85 Hz (January trials) and 87–90 Hz (May trials) (Fig. 2).

After the 15-min playback trial, fish were placed in a 5-gallon bucket for 15 min and then sacrificed by deep anesthetization in 0.025% benzocaine in seawater. Immediately after sacrifice, the standard length (SL) and weight were recorded for each animal, followed by transcardial perfusion to preserve the brain tissue. Fish were first perfused with ice cold teleost ringers followed by 4% paraformaldehyde in 0.1 M phosphate buffer (PB; pH 7.2). The brains were then harvested, post-fixed for 1 h, rinsed in 0.1 M PB (3X), and stored at 4°C in 0.1 M PB for <1 week. Next, brains were cryoprotected in 30%

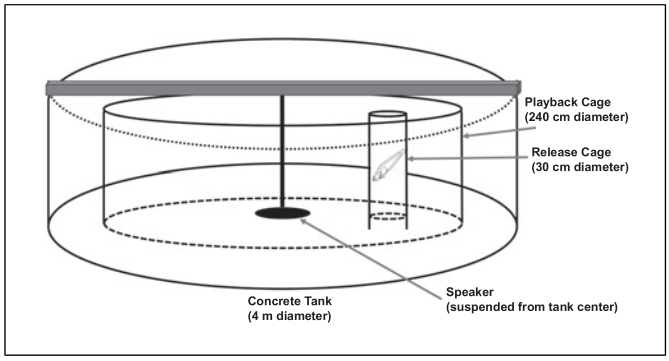

Fig. 1. Diagram of playback environment. Fish were placed in a release cage (30 cm diameter) situated on the outer edge of a mesh playback cage (240 cm diameter). A speaker suspended from the center of the tank broadcasts the male advertisement call (hum). The SPL at the release cage was calibrated to 130 dB re: 1 μPa.

sucrose-PB solution, embedded in cryogenic gel, and stored in a −80°C freezer until sectioning. Brains were sectioned on a cryostat in the transverse plane at 25 μm in two series, collected onto subbed slides, and stored at −80°C until labeling. For this experiment, one of two series was used for IHC. Finally, the testes of each fish were dissected and weighed and the gonadosomatic index (GSI) calculated ([gonad mass/body mass – gonad mass] × 100).

Immunohistochemistry

Slides were first brought to room temperature, then perimeter of each slide was traced with a hydrophobic pen and they were soaked 3X for 10 min in phosphate-buffered saline (PBS; pH 7.2), followed by a 1-h soak in a blocking solution made of PBS with 0.3% Triton X-100 and 5% normal donkey serum (PBS-DS; donkey serum from Jackson Immunolab, West Grove, PA, USA). Following the blocking procedure, slides were incubated for 16–17 h at room temperature in PBS-DS containing rabbit anti-pS6 (1:500; lot No. 16; cat No. 4858; Cell Signaling Technology, Danvers, MA, USA), sheep anti-TH (rate-limiting enzyme for DA synthesis;1:500; lot No. 3261295; cat No. AB1542; Millipore Sigma, Burlington, MA, USA), and mouse anti-Hu (1:1,000, lot No. 2105721; cat No. A-21271; Thermo Fisher Scientific, Waltham, MA, USA), which labels neurons [40–42]. After incubation, slides were briefly dipped and then rinsed in PBS +0.5% normal donkey serum 5X for 10 min. The slides were then incubated for 2 h at room temperature with PBS-DS containing donkey anti-rabbit conjugated to Alexa Fluor 568 (1:200; lot No. 2044343; cat No. A10042; Thermo Fisher Scientific) for pS6, anti-sheep Alexa Fluor 488 (1:200; lot No. 2079356; cat No. A-11015; Thermo Fisher Scientific) for TH, and anti-mouse Alexa Fluor 350 (1:200; lot No. 2045306; cat No. A10035; Thermo Fisher Scientific) for Hu. Finally, slides were dipped and rinsed 4X for 10 min in PBS before being coverslipped using ProLong Gold Antifade (without DAPI; cat No. P36930; Thermo Fisher Scientific) and allowed to cure for 48–72 h in a dark room. Once dried, the slides were sealed with nail polish and stored at 4°C. The IHC methods for TH and Hu were based on previous studies with midshipman [37, 38]. Phospho-S6 ribosomal protein (pS6) is a well-established marker used in studies across multiple teleost species [43–47, 49], including plainfin midshipman [50]. The specificity of the anti-pS6 primary antibody has been demonstrated in a prior study with *Astatotilapia burtoni*, a teleost fish [43], and confirmed for midshipman using Western blot analysis [50].

Image Acquisition

Images were obtained using a Nikon Eclipse 80i epifluorescence compound microscope (Nikon Instruments Inc., Melville, NY, USA) with a Prime 95B sCMOS camera (Teledyne Photometrics, Tuscon, AZ, USA) and NIS-Elements software (Nikon Instruments Inc.). For the three "auditory nuclei" (TSnc, CPc, AT), the regions were first identified using the DAPI filter to visualize neurons (Hu immunoreactive or Hu-ir cells) before being imaged using a 10X objective. Each photomicrograph was taken consecutively using DAPI and Texas Red filter sets, respectively. For the dopaminergic nucleus (TPp), the region was first identified using the GFP filter to visualize the dopaminergic TH immunoreactive (TH-ir) neurons before being imaged using a 10X objective. TH is the rate-limiting enzyme for DA synthesis and is a proxy for DA neuron activation [30]. Each photomicrograph was taken consecutively using GFP, Texas Red, and DAPI filter sets, respectively. For all regions examined, the exposure times and light levels were held constant for each channel across all conditions. Photomicrographs were then merged (TPp: "Batch RGB Merge" macro; auditory nuclei: "Batch RB Merge" modified macro) and analyzed using ImageJ (National Institutes of Health, Bethesda, MD, USA) [55]. For all auditory nuclei, images were first analyzed in the blue (Hu-ir) channel and the region of interest was outlined and isolated. The region of interest was then processed using a custom ImageJ macro "Object Count with Find Maxima" in the red channel to identify pS6 immunoreactive (pS6-ir) neurons [56]. The pS6-ir identified cells were then manually verified, ensuring only colocalized Hu-ir and pS6-ir cells were counted. The average number of pS6-ir cells per section in each auditory nucleus was recorded in each animal. For the TPp, the total

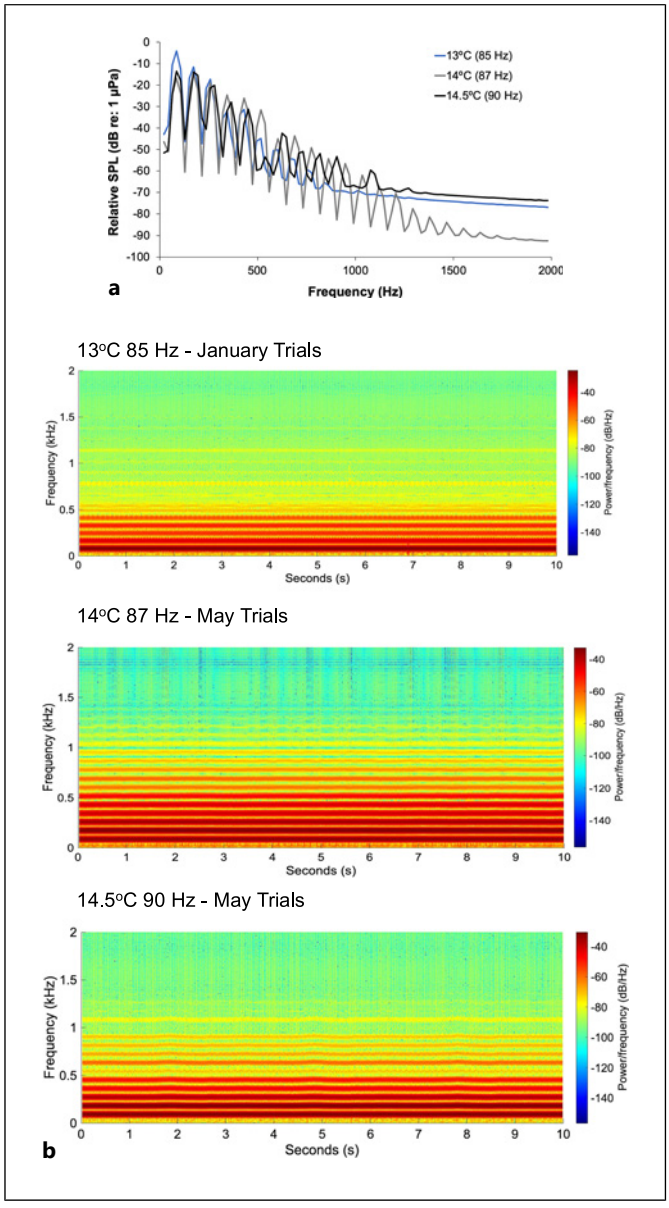

Fig. 2. Male midshipman advertisement calls. The three hums used in this study were recorded from calling male midshipman housed in outdoor tanks at the Friday Harbor Laboratory in three different water temperatures: 13°C (85 Hz), 14°C (87 Hz), 14.5°C (90 Hz). **a** Power spectral density curves generated using Audacity (v. 2.4.2, Free Software Foundation, Boston, MA, USA) with a Hamming window = 2,048. **b** Representative spectrograms (Hamming window of 2,048) generated using MATLAB (R2020a). SPL, sound pressure level.

number of TH-ir neurons were first counted using the custom "Manual Count" ImageJ macro in the green channel. pS6-ir neurons were then identified using the "Object Count with Find Maxima" macro and manually verified to confirm that only pS6-ir and TH-ir neurons were counted. For each animal, the percentage of total TH-ir neurons that also contained pS6 (hereafter referred to as colocalization) was then determined for each section.

Image Analysis and Neuroanatomy

Auditory Centers

The auditory pathway in the midshipman midbrain and forebrain has been well described [23, 24] and similar to previous work [38, 39], we imaged the following regions: the nucleus centralis of the TS (TSnc) of the midbrain and two forebrain regions: the compact division of central posterior nucleus of the thalamus (CPc) and the anterior tuberal nucleus of the ventral hypothalamus (AT) (Fig. 3). For all auditory regions, sections were sampled unilaterally on the right side throughout their entire extent. No left/right differences in hemispheric brain activation were predicted because fish were allowed to move freely above the underwater playback speaker as in Mohr et al. [39]. The regions were identified using landmarks from previously published neuroanatomical and physiological studies [e.g., 23, 24] and the sampling techniques were replicated from Petersen et al. [38] and Mohr et al. [39]. For the midbrain TSnc, similar to Peterson et al. [38], we sampled every fourth section (moving caudally to rostral), and we used five sections throughout the TSnc for analysis in all animals (except one individual in the reproductive control group for which four sections were analyzed). In the forebrain, both the CPc (identified as a wing-shaped band of cells lateral to the midline) and the AT receive projections from the TSnc and for each nucleus, we sampled three sections [38].

Dopaminergic TPp

Analysis of pS6-ir colocalization with the TH-ir neurons of the TPp began caudally with appearance of large pear-shaped dopaminergic (TH-ir) cells clustered medially to the medial forebrain bundle and extending ventrolaterally along the lateral border of the paraventricular organ (Fig. 3) [32, 36–38, 57]. TPp was analyzed serially for an average of 6.48 ± 0.75 (standard deviation [SD]) sections in the caudal to rostral direction until the disappearance of the large, pear-shaped TH-ir cells adjacent to the midline. There was no difference in the number of sections analyzed between groups (ANOVA $F(3, 20) = 1.47$; $p = 0.26$).

Statistics

For each auditory nucleus (TSnc, CPc, AT), the number of pS6-ir cells per section was averaged for each animal and compared between the four treatment groups

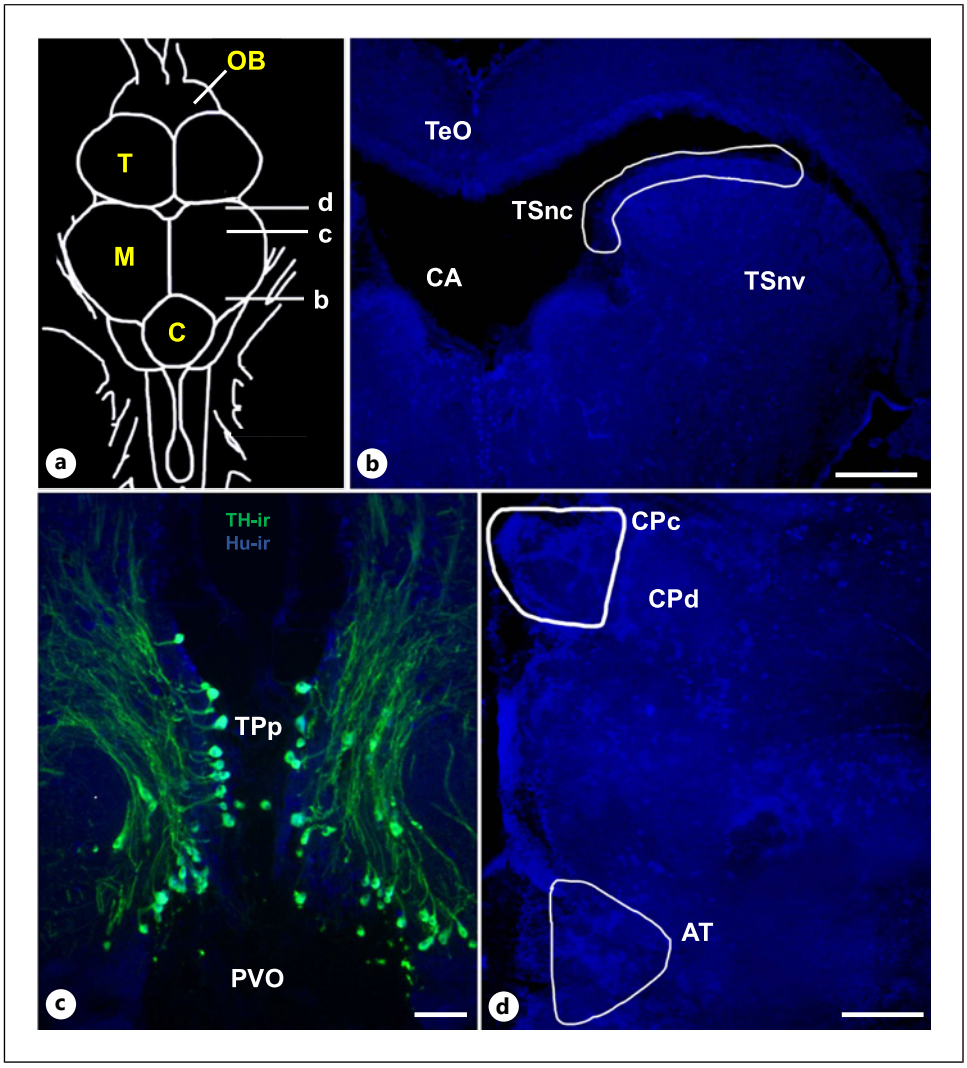

Fig. 3. Auditory and dopaminergic nuclei investigated in this study. Cytoarchitecture delineated with Hu protein immunoreactivity (Hu-ir), which labels all neuronal somata (blue). Auditory nuclei where a number of pS6-ir neurons were quantified are outlined in white. TH immunoreactivity (TH-ir), a marker for catecholamine synthesis, labels the dopaminergic periventricular posterior tuberculum (TPp) (green). **a** Drawing of the midshipman brain (dorsal view) indicating the relative levels of b–d. **b** Transverse section through the auditory midbrain containing the nucleus centralis of the TS (TSnc), outlined in white border. **c** Dopaminergic cells of the TPp labeled with anti-TH. **d** Section through the diencephalon containing the compact division of the central posterior nucleus in the auditory thalamus (CPc) and the anterior tuberal nucleus of the hypothalamus (AT) outlined by white border. CPd, diffuse division of the central posterior nucleus; M, midbrain; T, telencephalon (forebrain); C, cerebellum; OB, olfactory bulb; TSnv, torus semicircularis nucleus ventrolateralis; CA, cerebral aqueduct; TeO, optic tectum; PVO, paraventricular organ; scale bar = 500 μm, scale bar = 100 μm.

(nonreproductive control, NR hum, reproductive control, reproductive hum) using a two-way ANOVA (α set at 0.05) and Tukey HSD post hoc tests. For the dopaminergic TPp, average percent of TH-ir cells colocalized with pS6-ir per section was determined for each animal, and the percent data were arcsine transformed. A two-way ANOVA (α set at 0.05) and Tukey HSD post hoc tests were performed on the residuals. Shapiro-Wilk tests confirmed normal distribution of the data ($p > 0.05$). The Benjamini-Hochberg procedure was used to correct for multiple comparisons, with a false discovery rate of 0.25 [14, 36–39, 57, 58]. Statistical analyses were performed in RStudio (version 2023.06.0+421, PBC, Boston, MA, USA) [59]. All averages are reported as mean ± SD. In cases where the power is <0.8, we still report the significance; however, these results are associated with a higher likelihood of a type II error (Table 1).

Results

Of the 24 type I male midshipman used in the study, 11 were collected during the winter in January ($N = 6$ control; $N = 5$ hum) and 13 were collected during the spring in May ($N = 7$ control; $N = 6$ hum). In the NR (winter) condition, fish in the control group (ambient noise) had an SL of 24.38 ± 3.42 cm (mean ± SD), body mass of 203.63 ± 83.57 g, and a GSI of 1.76 ± 0.48. For the NR fish exposed to the male hum, the SL was 23.38 ± 5.77 cm, body mass was 181.67 ± 110.30 g, and GSI was 1.77 ± 0.87. In the reproductive (spring) condition, fish in the control group had an SL of 19.59 ± 4.73 cm, body mass of 110.26 ± 75.49 g, and GSI of 1.60 ± 0.65. The reproductive males exposed to the male hum had a SL of 18.12 ± 4.1 cm, body mass of 92.56 ± 61.0 g, and GSI of 2.09 ± 0.21. There were no differences between groups for any of the morphometric data analyzed (SL:

Table 1. Summary of results and statistical analyses

Treatment	TSnc	CPc	AT	TPp
	mean±SD (pS6-ir cells/section)			mean±SD % colocalization (pS6-ir and TH-ir)
NR control	18.57±3.56	22.55±9.77	76.05±11.35	21.91%±11.67%
NR hum	14.12±11.4	22.60±7.36	85.00±8.51	15.70%±16.61%
R control	19.06±8.88	38.71±10.03	75.99±11.23	7.98%±7.54%
R hum	34.52±10.17	67.03±12.11	108.78±15.81	38.52%±22.02%
ANOVA	TSnc	CPc	AT	TPp
	Test results, *p* value; power			
Treatment (hum vs. control)	$F(1,20) = 6.24$, ***p* = 0.021**; 0.40	$F(1, 20) = 19.12$, ***p* = 0.00029**; **0.84**	$F(1, 20) = 19.50$, ***p* = 0.00027; 0.85**	$F(1, 20) = 4.95$, ***p* = 0.038**; 0.33
Condition (R vs. NR)	$F(1, 20) = 6.61$, ***p* = 0.028**; 0.37	$F(1, 20) = 63.9$, ***p* < 0.0001**; **0.99**	($F(1, 20) = 4.79$, ***p* = 0.041**; 0.32	$F(1, 20) = 0.219$, ***p*** = 0.64
Treatment:condition	$F(1, 20) = 12.41$, ***p* = 0.0021**; **0.83**	$F(1, 20) = 11.94$, ***p* = 0.0025**; **0.81**	$F(1, 20) = 5.73$, ***p* = 0.027**; 0.47	$F(1, 20) = 8.78$, ***p* = 0.0077**; 0.67
Tukey HSD (B-H corrected)	TSnc	CPc	AT	TPp
	p value; power			
R hum:R control	**0.0075**; 0.48	**0.00075**; **0.81**	**0.0027**; **0.91**	**0.021**; **0.87**
R hum:NR control	**0.027**; 0.36	<**0.0001**; **0.99**	**0.0033**; **0.91**	0.44
R hum:NR hum	**0.0075**; 0.54	<**0.0001**; **0.99**	**0.042**; 0.67	0.17
R control:NR control	0.99	**0.026**; 0.45	0.99	0.60
R control:NR hum	0.99	0.068	0.86	0.99
NR control:NR hum	0.99	0.99	0.1	0.99

Bolded values indicate $p < 0.05$; power >0.8. R, reproductive; NR, non-reproductive; control, ambient control; B-H, Benjamini-Hochberg.

ANOVA $F(3, 20) = 2.37$; $p = 0.10$; weight: ANOVA $F(3, 20) = 2.35$; $p = 0.10$; GSI: ANOVA $F(3, 20) = 0.72$; $p = 0.55$). Although GSI is a measure of reproductive capacity, Sisneros et al. [60] reported that while mean GSI in type I males did not significantly differ between the reproductive and NR seasons, histological analysis revealed that testes from NR fish (collected via trawl in the winter) did not contain sperm, while the testes from reproductive fish (collected from nests) contained mature sperm. Furthermore, reproductive state can also be verified by the presence of robust sonic muscles, which develop in reproductive-state type I males [61, 62]. We are confident that the fish collected for this study in May (spring) represent the reproductive state as these fish were collected from nesting sites at low tide and had well-developed sonic muscles around the swim bladder [61, 62], while we observed diminished sonic muscles in NR males collected in January (winter).

In the midbrain, TSnc neurons were differentially activated based on both reproductive state and playback stimulus (ambient control vs. hum). A two-way ANOVA revealed a significant effect of both reproductive state ($F(1, 20) = 6.61$, $p = 0.028$) and playback stimulus ($F(1, 20) = 6.24$, $p = 0.021$), with a significant interaction between reproductive state and stimulus ($F(1, 20) = 12.41$, $p = 0.0021$) (Table 1). Reproductive (spring) males exposed to the advertisement call (hum) (mean ± SD pS6-ir cells/section; 34.52 ± 10.17) had significantly more pS6-ir TSnc neurons compared with reproductive males in the ambient control group (19.06 ± 8.88, $p = 0.0075$; Fig. 4) and the NR (winter) male groups (control:

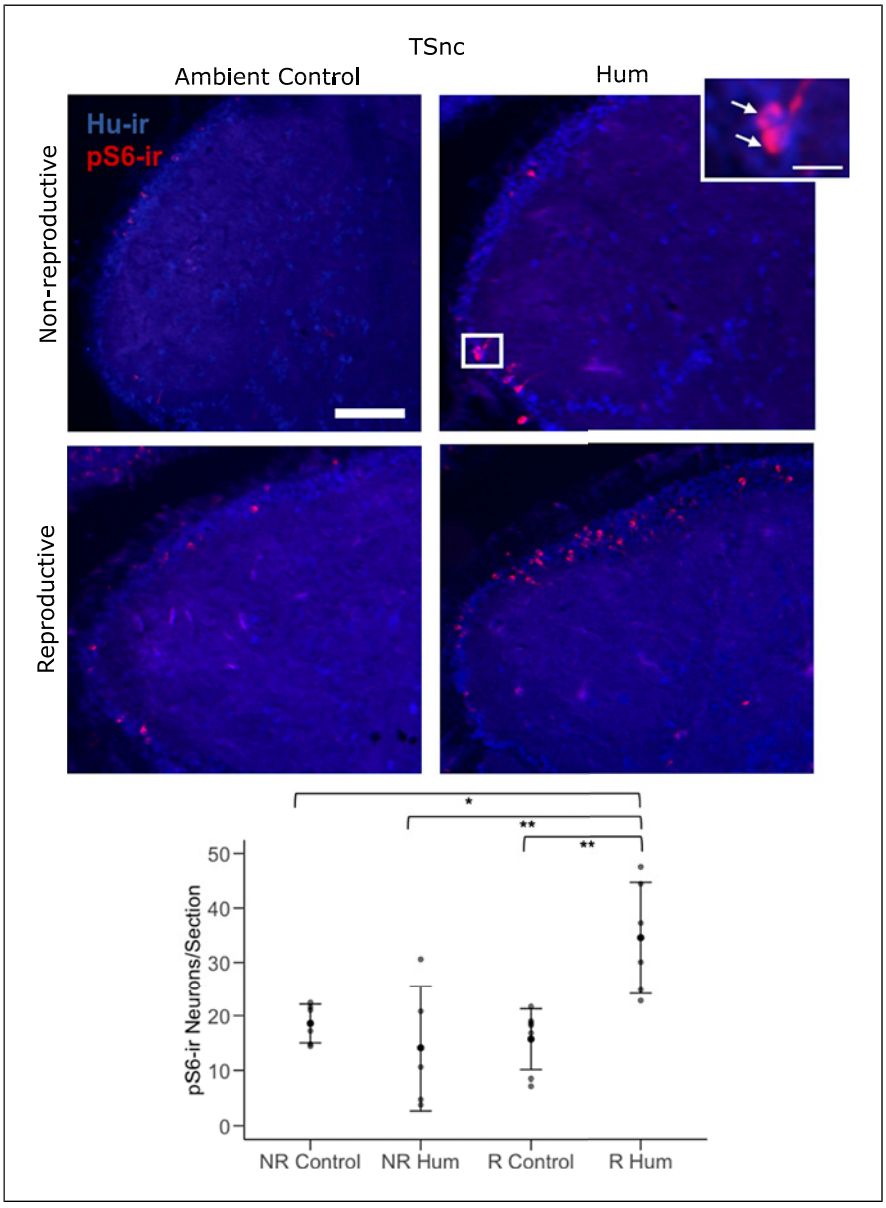

Fig. 4. Representative images of the nucleus centralis of the TS (TSnc) in the midbrain. Images are shown for all groups: NR (winter) control (ambient), NR hum (male advertisement call playback), reproductive (spring) control, and reproductive hum. Active neurons are identified by colocalization of neuron-specific anti-Hu (blue) and anti-pS6 (red). **Inset** White arrows indicate a representative sample of colocalized Hu-ir/pS6-ir neurons. Scatter plot represents the mean number of pS6-ir neurons per section ±SD. NR, non-reproductive; R, reproductive; *$p < 0.05$, **$p < 0.01$, ***$p < 0.001$; scale bar = 100 μm, scale bar = 25 μm.

18.57 ± 3.56, $p = 0.027$; hum: 14.12 ± 11.4, $p = 0.0075$; Fig. 4) (Table 1).

Forebrain nuclei were also differentially activated based on both reproductive state and playback stimulus, particularly in the CPc. For the CPc, a two-way ANOVA showed that there was a significant effect of both reproductive state ($F(1, 20) = 63.9$, $p < 0.0001$) and playback stimulus ($F(1, 20) = 19.12$, $p = 0.00029$), with a significant interaction between reproductive state and playback stimulus ($F(1, 20) = 11.94$, $p = 0.0025$) (Table 1). Reproductive males exposed to the hum had significantly more pS6-ir CPc neurons (67.03 ± 12.11, Fig. 5a) compared to reproductive males in the ambient control group (38.71 ± 10.03, $p = 0.00075$, Fig. 5a) and the NR male groups (ambient control: 22.55 ± 9.77, $p < 0.0001$; hum: 22.60 ± 7.36, $p < 0.0001$, Fig. 5a) (Table 1). Additionally, reproductive males in the ambient control group had more pS6-ir CPc neurons compared to both the NR male groups; however, after the Benjamini-Hochberg correction this difference was only significant when compared to the NR ambient control ($p = 0.026$; reproductive ambient control vs. NR hum: $p = 0.068$ [uncorrected: $p = 0.034$]) (Table 1).

In the AT, a two-way ANOVA showed a significant effect of reproductive state ($F(1, 20) = 4.79$, $p = 0.041$) and

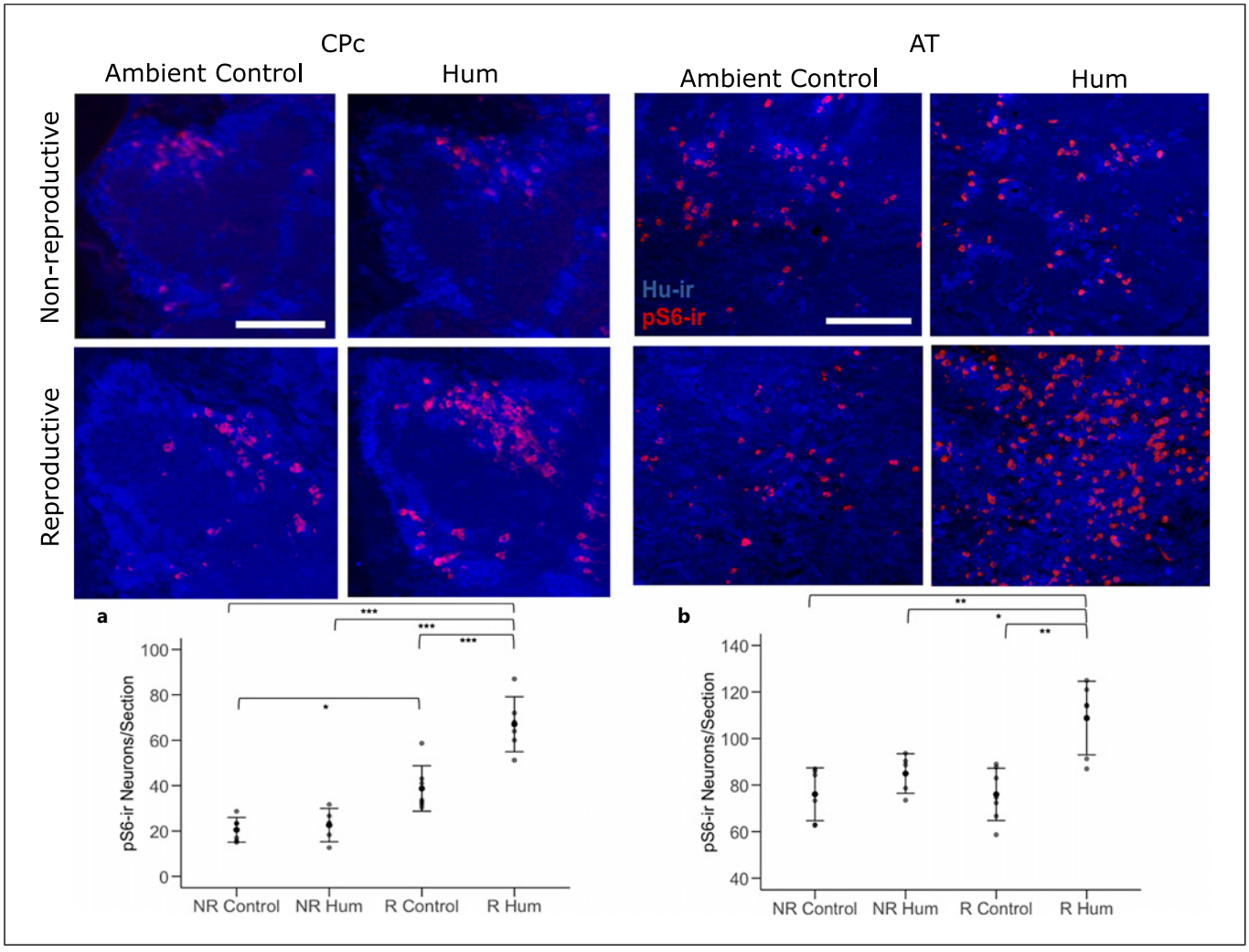

Fig. 5. Representative images of the compact division of the central posterior nucleus of the thalamus (CPc) and the anterior tuberal nucleus of the ventral hypothalamus (AT) in the forebrain from each of the four experimental groups: NR (winter) control (ambient), NR hum (male advertisement call playback), reproductive (spring) control, and reproductive hum. Active neurons are identified by colocalization of neuron-specific anti-Hu (blue) and anti-pS6 (red). Scatter plots (in **a**, **b**) represent the mean number of pS6-ir neurons per section ±SD. NR, non-reproductive; R, reproductive; *$p < 0.05$, **$p < 0.01$, ***$p < 0.001$; scale bar = 100 μm.

playback stimulus ($F(1, 20) = 19.50$, $p = 0.00027$), with an interaction effect between reproductive state and playback stimulus ($F(1, 20) = 5.73$, $p = 0.027$) (Table 1). Reproductive males exposed to the hum (108.78 ± 15.81, Fig. 5b) had significantly more pS6-ir AT neurons compared with reproductive males in the ambient control group (75.99 ± 11.23, $p = 0.0027$, Fig. 5b) and NR male groups (ambient control: 76.05 ± 11.35, $p = 0.0033$; hum: 85.00 ± 8.51, $p = 0.042$, Fig. 5b) (Table 1).

The dopaminergic neurons of the TPp were differentially activated based on playback condition. Although the TPp data are reported as percent colocalization of pS6-ir and TH-ir neurons, the percentage data were arcsine transformed for statistical tests. A two-way ANOVA on the residuals showed a significant effect of playback stimulus ($F(1, 20) = 4.95$, $p = 0.038$) and an interaction effect between reproductive state and playback stimulus ($F(1, 20) = 8.78$, $p = 0.0077$) (Table 1). Reproductive males exposed to the hum had a significantly higher percent colocalization (38.52% ± 22.02% [mean ± SD]) of pS6-ir and TH-ir neurons in the TPp compared to reproductive males in the ambient control group (7.98% ± 7.54%, $p = 0.021$, Fig. 6) (Table 1). Although the reproductive males exposed to the hum

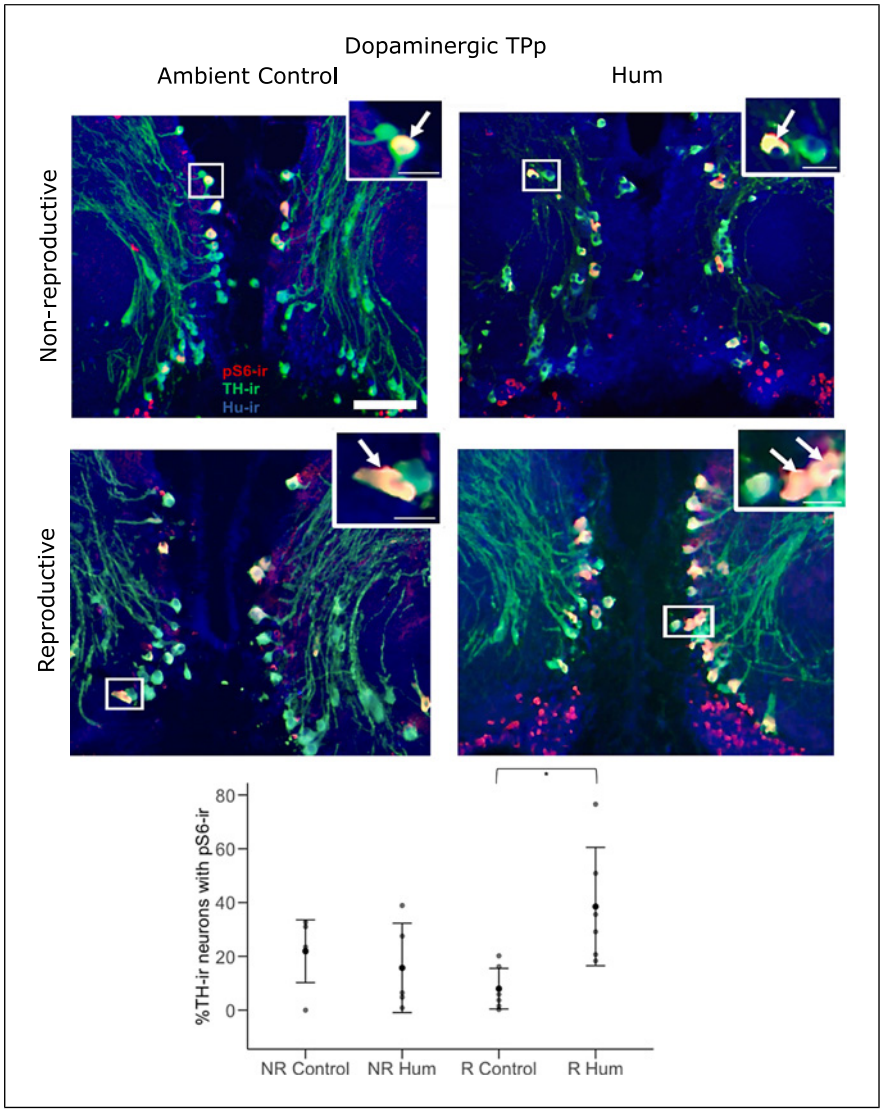

Fig. 6. Representative images of the dopaminergic periventricular posterior tuberculum (TPp) from each of the four experimental groups: NR (winter) control (ambient), NR hum (male advertisement call playback), reproductive (spring) control, and reproductive hum. Active neurons are identified by colocalization of dopaminergic neuron-specific anti-TH (green) and anti-pS6 (red). **Insets** White arrows indicate a representative sample of colocalized TH-ir/pS6-ir neurons. Scatter plot represents the mean percent colocalization ±SD; these percentage data were arcsine transformed for statistical tests. NR, non-reproductive; R, reproductive; *$p < 0.05$; scale bar = 100 μm, scale bar = 25 μm.

had higher activation in dopaminergic neurons compared to the NR groups (ambient control: 21.91% ± 11.67%, hum: 15.70% ± 16.61%), these differences were not significant ($p = 0.44$ and $p = 0.17$, respectively) (Table 1).

Discussion

The goal of this study was to compare neural activation in auditory midbrain, forebrain, and dopaminergic (DAergic) regions in type I male midshipman across two reproductive states (reproductive vs. NR) and under two auditory stimulus conditions (conspecific male advertisement call, i.e., the "hum" vs. no playback ambient control). Using phospho-S6 ribosomal protein (pS6) as a proxy for neural activity, we observed increased activation in the midbrain and forebrain auditory nuclei in reproductive male midshipman exposed to the conspecific male hum. Additionally, reproductive male midshipman exposed to the advertisement call showed increased pS6-ir colocalization in the TH-ir cells of the TPp, as a proxy for the activation of the DAergic neurons. Our results are consistent with previous studies in male type I [38] and female midshipman [39], which showed increased activation, as reported by a cFos antibody, in midbrain (TSnc), forebrain (AT, CPc), and DAergic (TPp) nuclei in response to male advertisement calls within the reproductive season. We also report differential

response of the DAergic TPp in reproductive male midshipman based on playback condition, which is consistent with previous work in midshipman [36–38].

Neural Activation in Midbrain Auditory Nuclei

The TS in anamniotes is thought to be homologous to the amniote inferior colliculus [63–65], a region associated with detection of social acoustic/communication signals [66]. In fishes and amphibians, the TS also plays a key role in processing auditory signals [38, 39, 67–70]. In anurans, several TS subdivisions are associated with auditory processing [71–74]. For instance, in a playback experiment using male túngara frogs (*Engystomops pustulosus*), Hoke et al. [72] found a relationship between egr-1 (an immediate early gene) activation in the TS and the behavioral salience of mating calls. In midshipman and other bony fishes, the nucleus centralis of the TS (TSnc) is the primary auditory center in the midbrain, receiving direct input from auditory regions of the hindbrain [3, 24, 75]. In the current study, we report increased activation of pS6 in TSnc neurons only in reproductive male midshipman exposed to the male hum. Our findings are consistent with the increased cFos (an immediate early gene) induction reported in the TSnc in reproductive male [38] and female [39] midshipman in response to the male hum but expand those initial findings to indicate this differential response to the advertisement call only occurs, at least in males, while in a reproductive state.

Neural Activation in Forebrain Auditory Nuclei

The compact division of the central posterior nucleus in the auditory thalamus (CPc) receives projections from the TSnc and is theorized to be involved in higher-order processing of auditory information in midshipman and other bony fishes [3, 24, 25]. We observed the highest pS6 activation in the CPc in reproductive male plainfin midshipman exposed to the male hum. Interestingly, the CPc was the only region where reproductive male midshipman in the ambient sound (reproductive control) group showed greater pS6 activation compared to NR control (i.e., winter state) individuals. Previous studies on male [38] and female [39] midshipman reported the highest cFos activation in groups exposed to the male hum, but all these fish were in reproductive condition. These results suggest that basal level of activity in the auditory thalamus is higher in the reproductive versus NR state. Based on single-unit recordings in the goldfish (*Carrasus auratus*), Lu and Fay [76] concluded that the CPc, in concert with the TSnc, is involved in discriminating acoustic signals. Northcutt [77] further theorized that the goldfish thalamic subdivisions likely integrate both auditory and visual sensory inputs to modulate sensorimotor response behavior. Together with these previous studies, our findings suggest that the increased activation in CPc and TSnc in reproductive male midshipman could reflect a reproductive-state-dependent enhancement in attention to/processing of conspecific signals.

Both male and female midshipman fish exhibit seasonal plasticity in the auditory periphery, resulting in decreased hearing thresholds (higher sensitivity) during the reproductive season [12–18]. Forlano et al. [14] reported that reproductive female midshipman had increased density of TH immunoreactive (TH-ir) fibers in the CPc compared to NR females, a seasonal difference not observed in the TSnc or AT. These fibers originate, at least in part, from the periventricular posterior tuberculum (TPp) [14, 32], and since they also project to the saccule of the inner ear, they are thought to modulate seasonal changes in hearing sensitivity at the periphery and auditory-driven reproductive behaviors. Mohr et al. [39] reported greater cFos activation in the CPc for female midshipman exposed to the male advertisement call compared with a heterospecific vocalization. Together with Mohr et al. [39], the results of the present study, which show higher pS6 activation in reproductive male midshipman, particularly in the male hum playback group (vs. ambient sound control), provide strong evidence that the CPc plays a crucial role in the processing of social acoustic signals.

The anterior tuberal nucleus (AT) also receives direct input from the TSnc and shares reciprocal connections with the CPc [3, 24, 25, 45]. We report that reproductive male midshipman exposed to the conspecific male advertisement call exhibited the highest pS6 activation. This finding is consistent with previous studies that reported increased cFos activation in the AT of male [38] and female [39] midshipman exposed to the male hum. Similar to TSnc and CPc, this effect was specific to the reproductive season.

Neural Activation in Dopaminergic Nuclei

Reproductive male midshipman exposed to the conspecific male advertisement call showed an increased percentage of dopaminergic (DAergic) neurons (expressing TH-ir) colocalized with pS6-ir. These results are consistent with the increased cFos-ir/TH-ir colocalized cells reported by Peterson et al. [38] in reproductive male midshipman exposed to the conspecific hum compared to controls. Similarly, Forlano et al. [36] showed that activation of TPp TH-ir neurons strongly correlated with the time gravid female midshipman attended to a speaker playing a synthetic hum. Interestingly, our results indicate

that differential responsiveness of TPp TH-ir neurons to the male advertisement call is only found in the reproductive state. These findings provide supporting evidence that DAergic neurons in the TPp (1) aid in coordinating motivated acoustic-driven reproductive behaviors [36] and (2) may play a role in modulating the seasonal plasticity of the central auditory system, especially in CPc [14].

Catecholamines have been associated with a variety of conspecific interactions, across vertebrate taxa including reproductive behaviors, male-male competition, and territory defense. For instance, DAergic neurons in the posterior tuberculum are associated with positive phonotaxic response behavior in female gray tree frogs (*Hyla versicolor*) responding to the male advertisement call [78]. Numerous studies in mammals and songbirds have implicated DA in male-motivated sexual behavior [79, 80] and vocal behavior in reproductive male zebra finches [81–83] and European starlings (*Sturnus vulgaris*) [84]. Furthermore, catecholamines have been linked with antagonistic behaviors in reproductive males. For instance, both DA and noradrenaline were increased in green anoles (*Anolis carolinensis*) responding to the presentation of actual [85] or simulated [86] male competitors. Sewall et al. [87] found an increase in noradrenaline metabolites in the auditory forebrain in male Lincoln's sparrows (*Melospiza lincolnii*) exposed to rival male songs.

Here, we show differential responsiveness of TH-ir neurons in the TPp in reproductive male midshipman based on playback condition. Most vertebrates (likely all Sarcopterygii, which includes tetrapods) possess an ascending DA system that originates in the midbrain; however, this system is not present in teleost fishes (largest group of bony fishes) [26, 28, 88]. In zebrafish (a species of teleost fishes), the large DAergic cells in the TPp project anteriorly to the ventral telencephalon [89, 90] and caudally to the hindbrain and spinal cord [89, 91–94]. In addition to directly innervating the forebrain CPc, the DAergic cells of the midshipman TPp also send projections to the saccule, one of the primary hearing end organs of the inner ear [32]. Forlano et al. [14] examined seasonal plasticity of these TPp innervations and reported increased TH-ir density in the CPc but decreased TH-ir density in the saccule in reproductive female midshipman. The results of the present study show that the reproductive males exposed to the hum had the highest percent co-localization of pS6-ir and TH-ir cells in the TPp, although these differences were not significant when compared with the NR condition. Differential activation of DAergic neurons in the TPp during the reproductive season may serve to increase auditory attention and coordinate directed behavioral responses to hearing male competitors [38].

In conclusion, research on midshipman suggests that the DAergic TPp (1) is preferentially activated by social acoustic signals [37, 38], (2) is the major contributor of DAergic input to both the central and peripheral auditory system [32, 33], and (3) has seasonally regulated DAergic innervation in females, in part by steroid hormones [14, 95]. The present study further supports a role of catecholamines, including DA, in modulating reproductive behaviors driven by the auditory system.

Acknowledgments

We would like to thank Kitty Brown and the UC Davis Bodega Marine Laboratory for logistical support. We also thank Jennifer Liang and the University of Minnesota Duluth Biology Department for use of their epifluorescence compound microscope and supplemental imaging was done at the University of St. Thomas thanks to Carolyn Zeiner. We would also like to thank Kelsie Murchy for sharing spectrogram code and Rob Mohr for providing feedback on an early draft of this manuscript.

Statement of Ethics

All experimental procedures were approved by the University of Washington Institutional Animal Care and Use Committee (protocol: 4079-06).

Conflict of Interest Statement

The authors declare no conflict of interests.

Funding Sources

This work was supported by the National Institutes of Health (Auditory Neuroscience Training Grant, T32: 4T32DC005361-15 to B.J.V.), the National Science Foundation (IOS-1456700 to J.A.S. and IOS-1456743 to P.M.F.), and Tow Professorship Award to P.M.F.

Author Contributions

Conceptualization, methodology, and writing – review and editing: B.J.V, J.P.T., N.R.L, J.A.S., and P.M.F.; formal analysis and funding acquisition: B.J.V, J.A.S., and P.M.F.; resources and supervision: J.A.S. and P.M.F; writing – original draft: B.J.V.

Data Availability Statement

All data generated or analyzed during this study are included in this article. Further inquiries can be directed to the corresponding author.

References

1 Grothe B, Carr CE, Casseday JH, Fritzsch B, Köppl C. The evolution of central pathways and their neural processing patterns. In: Manley GA, Fay RR, Popper AN, editors. Evolution of the Vertebrate Auditory System. Springer Handbook of Auditory Research. New York: Springer; 2004. Vol. 22. p. 289–359.

2 McCormick CA. Evolution of central auditory pathways in anamniotes. In: Webster DB, Popper AN, Fay RR, editors. The evolutionary Biology of hearing. New York: Springer; 1992.

3 McCormick CA. Anatomy of the central auditory pathways of fish and Amphibians. In: Fay RR, Popper AN, editors. Comparative hearing: fish and Amphibians. Springer handbook of auditory research. New York: Springer; 1999. Vol. 11. p. 155–217.

4 McCormick CA, Braford MR. Evolution of auditory system in anamniotes. In: Binder MD, Hirokawa N, Windhorst U, editors. Encyclopedia of neuroscience. Berlin: Springer; 2009.

5 Bass AH, McKibben JR. Neural mechanisms and behaviors for acoustic communication in teleost fish. Prog Neurobiol. 2003;69:1–26. https://doi.org/10.1016/s0301-0082(03)00004-2

6 Bass AH, Ladich F. Vocal-acoustic communication: from neurons to behavior. In: Webb JF, Fay RR, Popper AN, editors. Fish bioacoustics. New York: Springer; 2008. p. 253–78.

7 Arora HL. Observations on the habits and early life history of the batrachoid fish, Porichthys notatus Girard. Copeia. 1948;1948(2):89–93. https://doi.org/10.2307/1438409

8 Bass AH. Shaping brain sexuality. Am Sci. 1996;84(4):352–63.

9 Brantley RK, Bass AH. Alternative spawning tactics and acoustic signals in the plainfin midshipman fish, *Porichthys notatus* Girard (*teleosti, batrachoididae*). Ethology. 1994;96(3):213–32. https://doi.org/10.1111/j.1439-0310.1994.tb01011.x

10 DeMartini EE. Annual variations in fecundity, egg size and condition of the plainfin midshipman (*Porichthys notatus*). Copeia. 1990;1990:850–5. https://doi.org/10.2307/1446452

11 Balebail S, Sisneros JA. Long duration advertisement calls of nesting male plainfin midshipman fish are honest indicators of size and condition. J Exp Biol. 2022;225(8):jeb243889. https://doi.org/10.1242/jeb.243889

12 Sisneros JA, Bass AH. Seasonal plasticity of peripheral auditory frequency sensitivity. J Neurosci. 2003;23(3):1049–58. https://doi.org/10.1523/JNEUROSCI.23-03-01049.2003

13 Sisneros JA. Seasonal plasticity of auditory saccular sensitivity in the vocal plainfin midshipman fish, *Porichthys notatus*. J Neurophysiol. 2009;102(2):1121–31. https://doi.org/10.1152/jn.00236.2009

14 Forlano PM, Ghahramani ZN, Monestime CM, Kurochkin P, Chernenko A, Milkis D. Catecholaminergic innervation of central and peripheral auditory circuitry varies with reproductive state in female midshipman fish, *Porichthys notatus*. PLoS One. 2015;10(4):e0121914. https://doi.org/10.1371/journal.pone.0121914

15 Rohmann KN, Bass AH. Seasonal plasticity of auditory hair cell frequency sensitivity correlates with plasma steroid levels in vocal fish. J Exp Bio. 2011;214(Pt 11):1931–42. https://doi.org/10.1242/jeb.054114

16 Coffin AB, Mohr RA, Sisneros JA. Saccular-specific hair cell addition correlates with reproductive state-dependent changes in the auditory saccular sensitivity of a vocal fish. J Neuro. 2012;32(4):1366–76. https://doi.org/10.1523/JNEUROSCI.4928-11.2012

17 Bhandiwad AA, Whitchurch EA, Colleye O, Zeddies DG, Sisneros JA. Seasonal plasticity of auditory saccular sensitivity in "sneaker" type II male plainfin midshipman fish, *Porichthys notatus*. J Comp Physiol. 2017;203(3):211–22. https://doi.org/10.1007/s00359-017-1157-9

18 Lozier NR, Sisneros JA. Reproductive-state dependent changes in saccular hair cell density of the vocal male plainfin midshipman fish. Hear Res. 2019;383(383):107805. https://doi.org/10.1016/j.heares.2019.107805

19 Forlano PM, Sisneros JA, Rohmann KN, Bass AH. Neuroendocrine control of seasonal plasticity in the auditory and vocal systems of fish. Front Neuroendocrinol. 2015;37:129–45. https://doi.org/10.1016/j.yfrne.2014.08.002

20 McKibben JR, Bass AH. Behavioral assessment of acoustic parameters relevant to signal recognition and preference in a vocal fish. J Acoust Soc Am. 1998;104(6):3520–33. https://doi.org/10.1121/1.423938

21 Sisneros JA, Marchaterre MA, Bass AH. Otolithic endorgan projections of the inner ear in a vocal fish. Bioacoustics. 2002;12(2–3):137–9. https://doi.org/10.1080/09524622.2002.9753674

22 Sisneros JA, Forlano PM, Deitcher DL, Bass AH. Steroid-dependent auditory plasticity leads to adaptive coupling of sender and receiver. Science. 2004;305(5682):404–7. https://doi.org/10.1126/science.1097218

23 Bass AH, Marchaterre M, Baker R. Vocal-acoustic pathways in a teleost fish. J Neurosci. 1994;14(7):4025–39. https://doi.org/10.1523/JNEUROSCI.14-07-04025.1994

24 Bass AH, Bodnar DA, Marchaterre MA. Midbrain acoustic circuitry in a vocalizing fish. J Comp Neurol. 2000;419(4):505–31. https://doi.org/10.1002/(sici)1096-9861(20000417)419:4<505::aid-cne7>3.0.co;2-3

25 Goodson JL, Bass AH. Vocal-acoustic circuitry and descending vocal pathways in teleost fish: convergence with terrestrial vertebrates reveals conserved traits. J Comp Neurol. 2002;448(3):298–322. https://doi.org/10.1002/cne.10258

26 Schweitzer J, Lohr H, Filippi A, Driever W. Dopaminergic and noradrenergic circuit development in zebrafish. Dev Neurobiol. 2012;72(3):256–68. https://doi.org/10.1002/dneu.20911

27 Yamamoto K, Vernier P. The evolution of dopamine systems in chordates. Front Neuroanat. 2011;5:21. https://doi.org/10.3389/fnana.2011.00021

28 Smeets WJ, Gonzalez A. Catecholamine systems in the brain of vertebrates: new perspectives through a comparative approach. Brain Res Brain Res Rev. 2000;33(2–3):308–79. https://doi.org/10.1016/s0165-0173(00)00034-5

29 Meek J. Catecholamines in the brains of osteichthyes. In: Smeets W, Reiner A, editors. Phylogeny and development of catecholamine systems in the CNS of vertebrates. Cambridge University Press; 1994. p. 49–76.

30 Flatmark T. Catecholamine biosynthesis and physiological regulation in neuroendocrine cells. Acta Physiol Scand. 2000;168(1):1–17. https://doi.org/10.1046/j.1365-201x.2000.00596.x

31 Perelmuter JT, Forlano PM. Connectivity and ultrastructure of dopaminergic innervation of the inner ear and auditory efferent system of a vocal fish. J Comp Neurol. 2017;525(9):2090–108. https://doi.org/10.1002/cne.24177

32 Forlano PM, Kim SD, Krzyminska ZM, Sisneros JA. Catecholaminergic connectivity to the inner ear, central auditory, and vocal motor circuitry in the plainfin midshipman fish *Porichthys notatus*. J Comp Neurol. 2014;522(13):2887–927. https://doi.org/10.1002/cne.23596

33 Perelmuter JT, Forlano PM. Connectivity and ultrastructure of dopaminergic innervation of the inner ear and auditory efferent system of a vocal fish. J Comp Neuro. 2017;525(9):2090–108. https://doi.org/10.1002/cne.24177

34 O'Connell LA, Hofmann HA. The vertebrate mesolimbic reward system and social behavior network: a comparative synthesis. J Comp Neurol. 2011;519(18):3599–639. https://doi.org/10.1002/cne.22735

35 Wullimann MF, Mueller T. Teleostean and mammalian forebrains contrasted: evidence from genes to behavior. J Comp Neuro. 2004;475(2):143–62. https://doi.org/10.1002/cne.20183

36 Forlano PM, Licorish RR, Ghahramani ZN, Timothy M, Ferrari M, Palmer WC, et al. Attention and motivated response to simulated male advertisement call activates forebrain dopaminergic and social decision-making network nuclei in female midshipman fish. Integr Comp Biol. 2017;57(4):820–34. https://doi.org/10.1093/icb/icx053

37 Ghahramani ZN, Timothy M, Varughese J, Sisneros JA, Forlano PM. Dopaminergic neurons are preferentially responsive to advertisement calls and co- active with social behavior network nuclei in sneaker male midshipman fish. Brain Res. 2018;1701:177–88. https://doi.org/10.1016/j.brainres.2018.09.014

38 Petersen CL, Timothy M, Kim DS, Bhandiwad AA, Mohr RA, Sisneros JA, et al. Exposure to advertisement calls of reproductive competitors activates vocal-acoustic and catecholaminergic neurons in the plainfin midshipman fish, *porichthys notatus*. PLoS One. 2013;8(8):e70474. https://doi.org/10.1371/journal.pone.0070474

39 Mohr RA, Chang Y, Bhandiwad AA, Forlano PM, Sisneros JA. Brain activation patterns in response to conspecific and heterospecific social acoustic signals in female plainfin midshipman fish, *porichthys notatus*. Brain Behav Evol. 2018;91(1):31–44. https://doi.org/10.1159/000487122

40 Forlano PM, Deitcher DL, Myers DA, Bass AH. Anatomical distribution and cellular basis for high levels of aromatase activity in the brain of teleost fish: aromatase enzyme and mRNA expression identify glia as source. J N Sci. 2001;21(22):8943–55. https://doi.org/10.1523/JNEUROSCI.21-22-08943.2001

41 Zupanc GK, Hinsch K, Gage FH. Proliferation, migration, neuronal differentiation, and long-term survival of new cells in the adult zebrafish brain. J Comp Neuro. 2005;488(3):290–319. https://doi.org/10.1002/cne.20571

42 Hinsch K, Zupanc GK. Generation and long-term persistence of new neurons in the adult zebrafish brain: a quantitative analysis. Neuroscience. 2007;146(2):679–96. https://doi.org/10.1016/j.neuroscience.2007.01.071

43 Butler JM, Whitlow SM, Roberts DA, Maruska KP. Neural and behavioural correlates of repeated social defeat. Sci Rep. 2018;8(1):6818. https://doi.org/10.1038/s41598-018-25160-x

44 Butler JM, Herath EM, Rimal A, Whitlow SM, Maruska KP. Galanin neuron activation in feeding, parental care, and infanticide in a mouthbrooding African cichlid fish. Horm Behav. 2020;126:104870. https://doi.org/10.1016/j.yhbeh.2020.104870

45 Corrales Parada CD, Mayer U, Chagnaud BP. The dorsal part of the anterior tuberal nucleus responds to auditory stimulation in zebrafish (*Danio rerio*). eNeuro. 2024;11(7):ENEURO.0062-24.2024-24.2024. https://doi.org/10.1523/ENEURO.0062-24.2024

46 Dunlap KD, Teles MC, Oliveira RF. Social stimuli increase activity of adult-born cells in the telencephalon of zebrafish (Danio rerio). J Exp Biol. 2021;224(16):jeb242253. https://doi.org/10.1242/jeb.242253

47 Fischer EK, Westrick SE, Hartsough L, Hoke KL. Differences in neural activity, but not behavior, across social contexts in guppies, Poecilia reticulata. Behav Eco Sociobio. 2018;72(8):131. https://doi.org/10.1007/s00265-018-2548-9

48 Knight ZA, Tan K, Birsoy K, Schmidt S, Garrison JL, Wysocki RW, et al. Molecular profiling of activated neurons by phosphorylated ribosome capture. Cell. 2012;151(5):1126–37. https://doi.org/10.1016/j.cell.2012.10.039

49 Maruska KP, Butler JM, Field KE, Forester C, Augustus A. Neural activation patterns associated with maternal mouthbrooding and energetic state in an African cichlid fish. Neuroscience. 2020;446:199–212. https://doi.org/10.1016/j.neuroscience.2020.07.025

50 Tripp JA, Salas-Allende I, Makowski A, Bass AH. Mating behavioral function of preoptic galanin neurons is shared between fish with alternative male reproductive tactics and tetrapods. J Neurosci. 2020;40(7):1549–59. https://doi.org/10.1523/JNEUROSCI.1276-19.2019

51 Fenton TR, Gout IT. Functions and regulation of the 70 kDa ribosomal S6 kinases. Int J Biochem Cell Biol. 2011;43(1):47–59. https://doi.org/10.1016/j.biocel.2010.09.018

52 Ruvinsky I, Meyuhas O. Ribosomal protein S6 phosphorylation: from protein synthesis to cell size. Trends Biochem Sci. 2006;31(6):342–8. https://doi.org/10.1016/j.tibs.2006.04.003

53 Magnuson B, Ekim B, Fingar DC. Regulation and function of ribosomal protein S6 kinase (S6K) within mTOR signalling networks. Biochem J. 2012;441:1–21. https://doi.org/10.1042/BJ20110892

54 Bass AH, Clark CW. The physical acoustics of underwater sound communication. In: Simmons AM, Fay RR, Popper AN, editors. Acoustic communication. New York: Springer; 2003. p. 15–64.

55 Schneider C, Rasband W, Eliceiri K. NIH Image to ImageJ: 25 years of image analysis. Nat Methods. 2012;9(7):671–5. https://doi.org/10.1038/nmeth.2089

56 Timothy M, Forlano PM. A versatile macro-based neurohistological image analysis suite for ImageJ focused on automated and standardized user interaction and reproducible data output. J Neurosci Meth. 2019;324:108286. https://doi.org/10.1016/j.jneumeth.2019.04.009

57 Ghahramani ZN, Timothy M, Kaur G, Gorbonosov M, Chernenko A, Forlano PM. Catecholaminergic fiber innervation of the vocal motor system is intrasexually dimorphic in a teleost with alternative reproductive tactics. Brain Behav Evol. 2015;86(2):131–44. https://doi.org/10.1159/000438720

58 Butler JM, Maruska KP. The mechanosensory lateral line system mediates activation of socially relevant brain regions during territorial interactions. Front Behav Neurosci. 2016;10:93. https://doi.org/10.3389/fnbeh.2016.00093

59 R Core Team. R: a language and environment for statistical computing. Vienna, Austria: R Foundation for Statistical Computing; 2021. Available from: https://www.R-project.org/

60 Sisneros JA, Forlano PM, Knapp R, Bass AH. Seasonal variation of steroid hormone levels in an intertidal-nesting fish, the vocal plainfin midshipman. Gen Comp Endocrinol. 2004;136(1):101–16. https://doi.org/10.1016/j.ygcen.2003.12.007.

61 Sisneros JA, Alderks PW, Leon K, Sniffen B. Morphometric changes associated with the reproductive cycle and behaviour of the intertidal-nesting, male plainfin midshipman Porichthys notatus. J Fish Bio. 2009;74(1):18–36. https://doi.org/10.1111/j.1095-8649.2008.02104.x

62 Rogers LS, Lozier NR, Sapozhnikova YP, Diamond KM, Davis JL, Sisneros JA. Functional plasticity of the swim bladder as an acoustic organ for communication in a vocal fish. Proc Biol Sci. 2023;290(2013):20231839. https://doi.org/10.1098/rspb.2023.1839

63 Klug A, Bauer EE, Hanson JT, Hurley L, Meitzen J, Pollak GD. Response selectivity for species-specific calls in the inferior colliculus of Mexican free-tailed bats is generated by inhibition. J Neurophysiol. 2002;88(4):1941–54. https://doi.org/10.1152/jn.2002.88.4.1941

64 Suta D, Kvasnak E, Popelar J, Syka J. Representation of species-specific vocalizations in the inferior colliculus of the Guinea pig. J Neurophysiol. 2003;90(6):3794–808. https://doi.org/10.1152/jn.01175.2002

65 Syka J, Popelar J, Kvasnak E, Suta J, Jilek M. Processing of species-specific vocalizations in the inferior colliculus and medial geniculate body of the Guinea pig. In: Syka J, editor. Acoustical signal processing in the central auditory system. Boston: Springer; 1997. p. 431–41.

66 Casseday JH, Covey E. A neuroethological theory of the operation of the inferior colliculus. Brain Behav Evol. 1996;47(6):311–36. https://doi.org/10.1159/000113249

67 Crawford JD. Central auditory neurophysiology of a sound-producing fish: the mesencephalon of *Pollimyrus isidori* (*Mormyridae*). J Comp Physiol. 1993;172(2):139–52. https://doi.org/10.1007/BF00189392

68 Crawford JD. Feature-detecting auditory neurons in the brain of a sound producing fish. J Comp Physiol. 1997;180(5):439–50. https://doi.org/10.1007/s003590050061

69 Feng AS, Lin W. Differential innervation patterns of three divisions of frog auditory midbrain (*Torus semicircularis*). J Comp Neurol. 1991;306(4):613–30. https://doi.org/10.1002/cne.903060407

70 Hoke KL, Ryan MJ, Wilczynski W. Sexually dimorphic sensory gating drives behavioral differences in túngara frogs. J Exp Biol. 2010;213(Pt 20):3463–72. https://doi.org/10.1242/jeb.043992

71 Endepols H, Walkowiak W. Integration of ascending and descending inputs in the auditory midbrain of anurans. J Comp Physiol. 2001;186(12):1119–33. https://doi.org/10.1007/s003590000159

72 Hoke KL, Burmeister SS, Fernald RD, Rand AS, Ryan MJ, Wilczynski W. Functional mapping of the auditory midbrain during mate call reception. J Neurosci. 2004;24(50):11264–72. https://doi.org/10.1523/JNEUROSCI.2079-04.2004

73 Potter HD. Mesencephalic auditory region of the bullfrog. J Neurophysiol. 1965;28(6):1132–54. https://doi.org/10.1152/jn.1965.28.6.1132
74 Wilczynski W. Afferents to midbrain auditory center in the bullfrog *Rana catesbeiana*. J Comp Neurol. 1981;198(3):421–33. https://doi.org/10.1002/cne.901980304
75 Bass AH, Rose GJ, Pritz MB. Auditory midbrain of fish, amphibians and reptiles: model systems for understanding auditory function. In: Winer JA, Schreiner CE, editors. The inferior colliculus. New York: Springer; 2005. p. 459–92.
76 Lu Z, Fay RR. Acoustic response properties of single neurons in the central posterior nucleus of the thalamus of the goldfish, *Carassius auratus*. J Comp Physiol. 1995;176(6):747–60. https://doi.org/10.1007/BF00192623
77 Northcutt RG. Connections of the lateral and medial divisions of the goldfish telencephalic pallium. J Comp Neurol. 2006;494(6):903–43. https://doi.org/10.1002/cne.20853
78 Endepols H, Schul J, Gerhardt HC, Walkowiak W. 6-hydroxydopamine lesions in anuran amphibians: a new model system for Parkinson's disease? J Neurobiol. 2004;60(4):395–410. https://doi.org/10.1002/neu.20047
79 Balfour ME, Lei Y, Coolen LM. Sexual behavior and sex-associated environmental cues activate the mesolimbic system in male rats. Neuropsyhopharmacol. 2004;29:718–30.
80 Hull EM, Du J, Lorrain DS, Matuszewich L. Extracellular dopamine in the medial preoptic area: implications for sexual motivation and hormonal control of copulation. J Neurosci. 1995;15(11):7465–71. https://doi.org/10.1523/JNEUROSCI.15-11-07465.1995
81 Goodson JL, Kabelik D, Kelly AM, Rinaldi J, Klatt JD. Midbrain dopamine neurons reflect affiliation phenotypes in finches and are tightly coupled to courtship. Proc Natl Acad Sci USA. 2009;106(21):8737–42. https://doi.org/10.1073/pnas.0811821106
82 Hara E, Kubikova L, Hessler NA, Jarvis ED. Role of the midbrain dopaminergic system in modulation of vocal brain activation by social context. Eur J Neurosci. 2007;25(11):3406–16. https://doi.org/10.1111/j.1460-9568.2007.05600.x
83 Sasaki A, Sotnikova TD, Gainetdinov RR, Jarvis ED. Social context-dependent singing-regulated dopamine. J Neurosci. 2006;26(35):9010–4. https://doi.org/10.1523/JNEUROSCI.1335-06.2006
84 Heimovics SA, Salvante KG, Sockman KW, Riters LV. Individual differences in the motivation to communicate relate to levels of midbrain and striatal catecholamine markers in male European starlings. Horm Behav. 2011;60(5):529–39. https://doi.org/10.1016/j.yhbeh.2011.08.001
85 Ling TJ, Summers CH, Renner KJ, Watt MJ. Opponent recognition and social status differentiate rapid neuroendocrine responses to social challenge. Physiol Behav. 2010;99(5):571–8. https://doi.org/10.1016/j.physbeh.2010.01.025
86 Watt MJ, Forster GL, Korzan WJ, Renner KJ, Summers CH. Rapid neuroendocrine responses evoked at the onset of social challenge. Physiol Behav. 2007;90(4):567–75. https://doi.org/10.1016/j.physbeh.2006.11.006
87 Sewall KB, Caro SP, Sockman KW. Song competition affects monoamine levels in sensory and motor forebrain regions of male lincoln's sparrows (*Melospiza lincolnii*). PLoS One. 2013;8(3):e59857. https://doi.org/10.1371/journal.pone.0059857
88 Carrera I, Anadon R, Rodrıguez-Moldes I. Development of tyrosine hydroxylase-immunoreactive cell populations and fiber pathways in the brain of the dogfish *Scyliorhinus canicula*: new perspectives on the evolution of the vertebrate catecholaminergic system. J Comp Neurol. 2012;520(16):3574–603. https://doi.org/10.1002/cne.23114
89 Kastenhuber E, Kratochwil CF, Ryu S, Schweitzer J, Driever W. Genetic dissection of dopaminergic and noradrenergic contributions to catecholaminergic tracts in early larval zebrafish. J Comp Neurol. 2010;518(4):439–58. https://doi.org/10.1002/cne.22214
90 Yamamoto K, Ruuskanen JO, Wullimann MF, Vernier P. Differential expression of dopaminergic cell markers in the adult zebrafish forebrain. J Comp Neurol. 2011;519(3):576–98. https://doi.org/10.1002/cne.22535
91 Rink E, Wullimann MF. The teleostean (zebrafish) dopaminergic system ascending to the subpallium (striatum) is located in the basal diencephalon (posterior tuberculum). Brain Res. 2001;889(1–2):316–30. https://doi.org/10.1016/s0006-8993(00)03174-7
92 Ma PM. Catecholaminergic systems in the zebrafish IV. Organization and projection pattern of dopaminergic neurons in the diencephalon. J Comp Neurol. 2003;460(1):13–37. https://doi.org/10.1002/cne.10544
93 McLean DL, Fetcho JR. Ontogeny and innervation patterns of dopaminergic, noradrenergic, and serotonergic neurons in larval zebrafish. J Comp Neurol. 2004;480(1):38–56. https://doi.org/10.1002/cne.20280
94 Tay TL, Ronneberger O, Ryu S, Nitschke R, Driever W. Comprehensive catecholaminergic projectome analysis reveals single-neuron integration of zebrafish ascending and descending dopaminergic systems. Nat Commun. 2011;2:171. https://doi.org/10.1038/ncomms1171
95 Perelmuter JT, Hom KN, Mohr RA, Demis L, Kim S, Chernenko A, et al. Testosterone treatment mimics seasonal downregulation of dopamine innervation in the auditory system of female midshipman fish. Integr Comp Biol. 2021;61(1):269–82. https://doi.org/10.1093/icb/icab070