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# Effects of Retinal Gonadotropin-Releasing Hormone on Reproductive Behavior and Retinal Activity in the Cichlid Fish, Astatotilapia burtoni

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# EFFECTS OF RETINAL GONADOTROPIN-RELEASING HORMONE ON REPRODUCTIVE BEHAVIOR AND RETINAL ACTIVITY IN THE CICHLID FISH, ASTATOTILAPIA BURTONI

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# ABSTRACT

Many animals use visual signals to evade predators, forage for food, and communicate with conspecifics. Some animals that shift into and out of breeding stages, such as fish, can modulate their visual sensitivity to better adapt to changing internal and external conditions. Previous work in our lab demonstrated that females of the cichlid fish *Astatotilapia burtoni* have improved visual sensitivity when they enter reproductive readiness. However, little is known about *how* fish and vertebrates more broadly modulate visual sensitivity across their reproductive cycle. We hypothesized that the hormone and neuromodulator gonadotropin-releasing hormone (GnRH) could modulate visual sensitivity because GnRH is a regulator of the reproductive cycle, and some GnRH neurons in the brain project to the retina. To examine the role of GnRH in modulating retinal activity in ovulated female *A. burtoni*, I first used calcium imaging to observe neural activity. Bath application of GnRH on live retina slices decreased outer nuclear layer fluorescence but increased inner nuclear layer and ganglion cell layer fluorescence. Antide, a GnRH receptor antagonist, following the GnRH had little effect. These data illustrate that GnRH can affect retinal activity. I also injected ovulated female eyes with GnRH or antide and measured changes in reproductive behavior in the presence of a courting male. Sham, GnRH, or antide eye injections in already ovulated females did not affect male courtship behavior, but antide eye injections reduced female responsiveness to courtship overall. In trials where spawning did not happen, GnRH-injected females often chose to intentionally avoid courtship whereas antide-injected females were more likely to not respond to courtship at all. This suggests that the GnRH system in the retina may ultimately impact reproductive decision-making. Lastly, the

retinas of the fish from the behavior study were labeled for the neural activation marker, pS6. Higher quantities of cells expressing pS6 were observed in GnRH eye-injected females across all quantified layers (inner nuclear, amacrine cell, and ganglion cell layer). Overall, this study is the first to integrate behavioral and neural activation analyses to show the importance of GnRH in regulating retinal activity and reproductive communication in fish.

# INTRODUCTION

In a world filled with friendly neighbors and deadly predators, animals rely on visual information to integrate their rapidly fluctuating physical and social environments to behave accordingly. Across taxa, visual signals such as behavior displays and coloration patterns transmit essential information about an animal's identity, sex, and reproductive and social status (Bell & Zamudio, 2012; Endler, 1980; Endler et al., 2005; Hughes et al., 2015). This visual communication is crucial for reproductive behaviors ranging from mate-choice to copulation, thereby directly influencing reproductive success. Examining the role of vision in reproduction is important for better understanding how sexual selection contributes to the evolution of species (Clutton-Brock, 2007; Seehausen et al., 1999).

In species that cycle in and out of breeding condition, fluctuations in conserved sex steroids and gonadotropins can influence context-specific adaptive behaviors and sensory processing. For example, female midshipman fish and many birds have increased circulating sex-steroid levels in the breeding season, which heightens peripheral auditory reception and central auditory processing, together improving their ability to hear and better respond to male courtship calls (Maney & Pinaud, 2011; Sisneros, 2009). Similar reproductive state-dependent auditory plasticity is seen in amphibians (Goense & Feng, 2005) and humans (Guimaraes et al., 2006). Furthermore, sex hormones across the estrous cycle modulate the activity of olfactory receptor neurons (Dey et al., 2015; Kanageswaran et al., 2016) and likely the central processing of olfaction (Kass et al., 2017) in female mice. These studies show that reproductive hormones can mediate central and peripheral sensory plasticity of auditory

and olfactory systems. However, despite the priority of visual information in many species, the mechanisms responsible for visual plasticity, especially within the retina, remain surprisingly unexplored across all taxa.

While sex steroids have a role in sensory plasticity, gonadotropin-releasing hormone (GnRH) is a strong candidate modulator of visual plasticity in the retina because of its known neuromodulatory function and proximity with the retina. GnRH exists as several different isoforms across vertebrates (Table 1). GnRH1 is classically known for its role as an upstream regulator of reproductive state and behavior by regulating the hypothalamic-pituitary-gonadal (HPG) axis (Moss & McCann, 1973; Pfaff, 1973) and is well-conserved across vertebrates. GnRH1 is produced and released from the hypothalamus and sent to the pituitary gland to stimulate gonadotropin (follicle stimulating hormone and luteinizing hormone) release, which travel in the bloodstream to affect gamete and sex-steroid production. GnRH is also found outside the hypothalamus (Ogawa et al., 2022; Umatani & Oka, 2019). GnRH2 is also remarkably conserved across all vertebrate species and is a known neuromodulator in many midbrain and hindbrain regions (Desaulniers et al., 2017). Unlike the hypothalamic GnRH1 neurons that project to the pituitary, GnRH1 and 3 neurons (species dependent) within the terminal nerve ganglia (i.e., nucleus olfactoretinalis) project directly to many regions throughout the brain including the telencephalon and thalamus (forebrain), tegmentum (midbrain), medulla (hindbrain), the olfactory bulbs (Munz et al., 1981; Oka & Matsushima, 1993; Schwanzel-Fukuda & Silverman, 1980), and importantly, to the contralateral retina through the optic nerves (Munz et al., 1982). GnRH-immunoreactive (GnRH-ir) axons are in the optic nerves or retina of amphibians (Wirsig-Wiechmann,

1993), reptiles (Medina et al., 2005), birds (Fukuda et al., 1982), mammals (Wirsig-Wiechmann & Wiechmann, 2002), and relative to other vertebrates, the highest densities of GnRH-ir fibers are in fish retina (GnRH3, primarily found in fish) (Munz et al., 1982; Munz et al., 1981; Stell et al., 1984). In fish, GnRH3 axons from the brain terminate near retinal dopaminergic interplexiform cells, so GnRH has been proposed to affect retinal dopaminergic activity (Grens et al., 2005; Umino & Dowling, 1991). Further, GnRH receptors (GnRHRs) are expressed in the retina of mammals (Skinner et al., 2009; Zoroquiain et al., 2015), amphibians (Chen & Fernald, 2008), and fish (Corchuelo et al., 2017; Grens et al., 2005). For example, GnRH directly affects firing activity of rat hippocampal neurons (Wong et al., 1990), central auditory processing neurons (Maruska & Tricas, 2011), olfactory neurons and processing centers (Kawai et al., 2009), and the activity of a few retinal cell types including horizontal and ganglion cells (Stell et al., 1984; Umino & Dowling, 1991). While there's strong evidence for GnRH as a neuromodulator of sensory systems, an integrative study linking the reproductive cycle and the modulatory effects of GnRH on sensory processing in the retina is missing.

Table 1. Different GnRH amino acid sequences. Positions 1-4, 6, and 9-10 are conserved across vertebrates – bold amino acids show genetic divergence. Data from (Millar, 2005).

| GnRH forms | Amino Acid Sequence |
|---|---|
| **GnRH1** | pGlu - His - Trp - Ser - **Tyr** - Gly - **Leu** - **Arg** - Pro - Gly (-$NH_2$) |
| **GnRH2** | pGlu - His - Trp - Ser - **His** - Gly - **Trp** - **Tyr** - Pro - Gly (-$NH_2$) |
| **GnRH3** | pGlu - His - Trp - Ser - **Tyr** - Gly - **Trp** - **Leu** - Pro - Gly (-$NH_2$) |

*Astatotilapia burtoni*, an African cichlid fish species endemic to Lake Tanganyika, is an ideal model for studying reproductive visual plasticity because they rely heavily on visual communication and have well-characterized reproductive behaviors and hormonal profiles (Figure 1). *A. burtoni* males can rapidly and reversibly switch between a subordinate and dominant phenotypic state. If not socially suppressed by another male, a subordinate male will ascend to the dominant state where he increases visual signaling to cohorts by intensifying coloration and actively courting females with behaviors such as quivers (close-range body vibrating), waggles (exaggerated swimming motion), leads (swimming towards his spawning territory), dances (long-range body vibrating and spinning in spawning territory), and chases. Reproductively-ready (ovulated) female *A. burtoni* are typically very receptive to male courtship, and upon accepting a male courtship attempt, she will follow a male into his spawning territory, spawn with him, and carry fertilized eggs in her mouth for two weeks through fry development (mouthbrooding). After fry release, she will recover for a few weeks, become gravid with developed eggs, and ovulate again to continue the reproductive cycle (Figure 1). Using electroretinograms to measure retinal activity, our lab has shown

that when females ovulate and are receptive to male courtship, their visual sensitivity to a wide range of colors improves dramatically (Butler et al., 2019). The heightened visual sensitivity during ovulation also correlates with an increase in retinal GnRH receptor *mRNAs* (*gnrhr1* and *gnrhr2*). Further, retinal *gnrhr2 mRNA* also positively correlated with positive female responses to male courtship behavior. GnRH3 can activate both GnRHR1 and GnRHR2 receptor types (Robison et al., 2001), therefore GnRH3 in the retina stands to affect retinal activity. However, direct evidence showing which retinal cells layers are affected by GnRH, the degree to which retinal activity is affected by GnRH, and how GnRH may lead to adaptive behaviors and better mate-choice decisions is unknown.

The overall purpose of this study was to test the hypothesis that GnRH acts as a mediator of reproductive state-dependent visual plasticity. The specific goals were to use reproductively-ready female *A. burtoni* to 1) qualitatively observe GnRH and GnRHR antagonist (antide) effects on activity in live retinal tissue, 2) quantify layer-specific changes in retinal activity in response to modulated retina GnRH levels, and 3) quantify behavioral effects of different retinal GnRH levels during reproductive encounters. Examining how reproductive state modulates vision is important for understanding how animals' sensory perception adapts to varying behavioral contexts, impacting reproductive success.

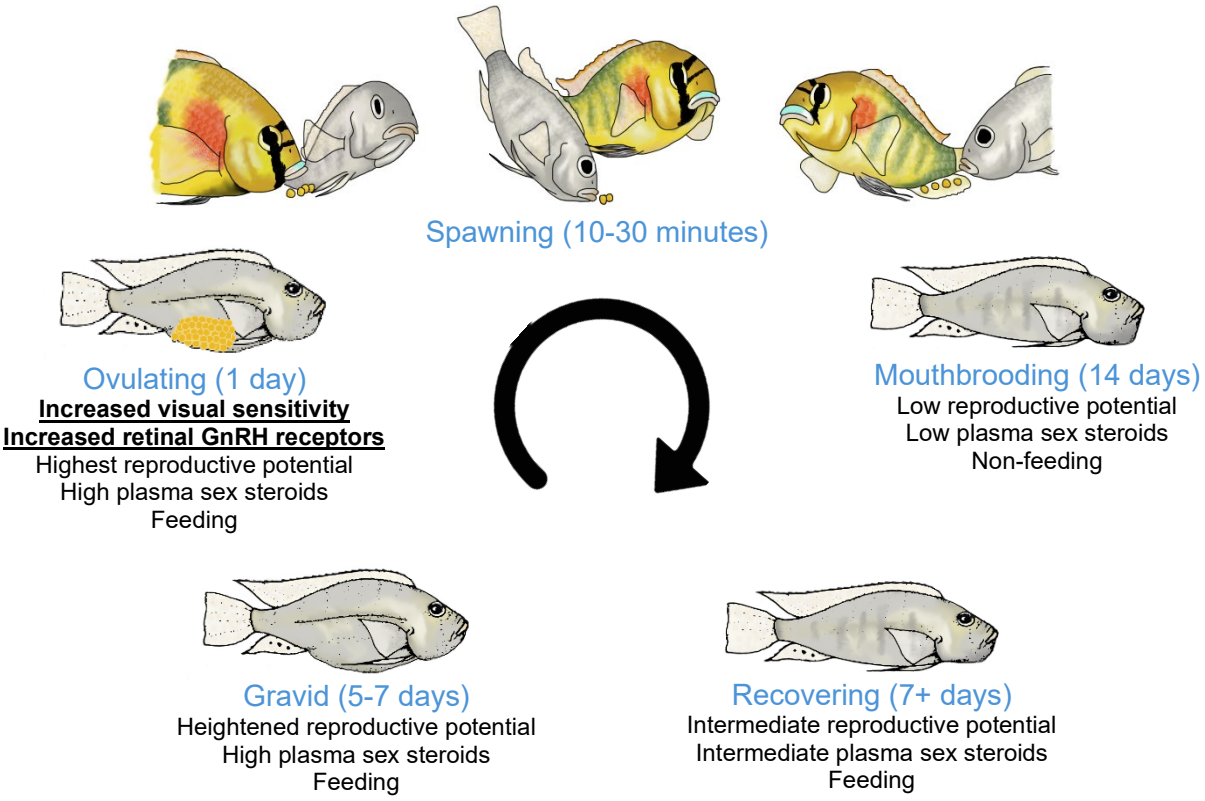


Figure 1. Reproductive sequence of *A. burtoni*: female reproductive states, spawning, and timelines. Reproductive endeavors in females begin with the production of ova to enter the gravid state. During ovulation, matured ova will collect near the genital papilla in preparation for release during spawning, and the jaw will distend to provide room for eggs throughout spawning and mouthbrooding. Also, she is actively courted by a dominant male. All females in this study were ovulated, and these three outward characteristics of ovulated females allowed for visual identification prior to use. Spawning begins when the female follows the male to his spawning territory and deposits her eggs on the substrate below. She immediately collects these eggs into her mouth and nips at the male's egg spots near his anal fin while he fertilizes the eggs. The female then enters her mouthbrooding stage during fry development and releases the fry after 14 days of starvation. After sufficient recovery, the female begins producing ova as she enters the gravid state again and the cycle repeats.

# MATERIALS & METHODS

## Animals

Adult African cichlid fish *Astatotilapia burtoni* were lab-bred from a stock that was derived from Lake Tanganyika, Africa (Fernald & Hirata, 1977)and housed in conditions similar to their natural habitat (pH ~8.0, temperature ~28°C, and a 12 hour light:12 hour dark cycle). Tank bottoms were covered in brown gravel and terracotta pot halves provided spawning territories. Fish were fed with cichlid algae flakes every morning. All experiments were conducted in accordance with guidelines stated in the National Institutes of Health Guide for the care and use of Laboratory Animals, and experimental protocols were approved by the LSU Institutional Animal Care and Use Committee (IACUCAM-23-057).

All females collected in this study were ultimately anesthetized in ice cold cichlid water and sacrificed by rapid cervical transection before whole retina were collected (tissue dissection, collection, and treatment described below). Standard length (length from snout to distal caudal peduncle), total length (length from snout to end of tail), body mass, and gonad mass were all measured at euthanizing. Gonadosomatic index (GSI = $\frac{Ovary\ Mass}{Body\ Mass} * 100$) was calculated to assign reproductive state (GSI>7 = gravid/ovulated). Ovulated females could also be distinguished from non-ovulated gravid females by their slightly distended jaw (in preparation for mouthbrooding) and protruding urogenital papilla. Reproductive state was further confirmed by the presence of active male courtship in the breeding community tanks immediately before use.

**Calcium imaging of live retinal tissue from ovulated females in response to GnRH or antide**

To examine the role of GnRH on activity within ovulated female retina, ovulated females of about the same size (38-42 mm standard length) were collected from community tanks on the morning of the experiment. Fish were anesthetized in ice water, and immediately euthanized with rapid cervical transection. The eyes were removed from the head and one retina was isolated and flat-mounted inside a 2% agarose gel block (the other eye was used for tyrosine hydroxylase immunohistochemistry, detailed below). While the agarose gel hardened, the calcium indicator solution was prepared. To create the stock solution, 50 µg Fluo-4 AM (Invitrogen, AM: acetoxymethyl, increases membrane permeability) was vortexed in 44 µL Dimethylsulfoxide (DMSO, VWR) and combined with 9 µL of Pluronic F-127 (Invitrogen) to facilitate cell loading. Next, 50 µL of the stock solution was mixed with 14.3 mL Ringer's solution (133 mM NaCl, 2.5 mM KCl, 2 mM $CaCl_2 \cdot 2H_2O$, 1.5 mM $NaH_2PO_4$, 1.5 mM $MgCl_2 \cdot 6H_2O$, 10 mM HEPES, 0.3 mM ascorbic acid, 10 mM D-glucose, pH 7.4 (Giarmarco et al., 2018), all reagents sourced from Sigma) for the final working calcium indicator solution of 3 µM fluo-4 AM. Every Ringer's batch was made fresh before each experiment. 200 µm retinal slices were prepared using a vibratome (Leica VT1200). To aid with a consistent location of imaging, only retinal slices that included the optic disc were selected to proceed, and imaging was later conducted within 600 µm of the optic disc. The retinal slices were then bathed in the fluorescent calcium indicator solution for 60-90 minutes in the dark at room temperature.

One eye was imaged per experiment. Individual retinal slices were placed under the microscope in the recording chamber and weighed down with silver weights. Retinal

slices were bathed in flowing Ringer's solution Each experiment's batch of Ringer's was used to make the respective GnRH (Sigma) and antide (Cayman) solutions. Antide is a third-generation GnRHR competitive antagonist that has the advantage of inducing minimal histamine release (Bliss et al., 2010; Leal, 1991; Li et al., 1994). Brightfield imaging under 4x objective lens was briefly used to locate either the outer nuclear layer, amacrine cell layer (for the entire study, "amacrine cell layer" is defined as the inner 1/3 of the inner nuclear layer), or ganglion cell layer. With 488 nm excitation and 520 nm emission filters images were taken at 2Hz (scope: Olympus BX50; software: Live Acquisition with Offline Analysis, TILL Photonics, FEI) under 40x objective lens (Olympus LUMPlanFL N, 40x/0.80w). A pilot study bathing 10, 125, or 1000 nM GnRH for 2 minutes showed that 125 nM GnRH caused effects in the retina (Figure 2) and no effects were seen with 10 or 1000 nM. 125 nM GnRH3 is also known to be within the range of sensitivity of neurons in brain auditory processing centers of *A. burtoni* (Maruska & Tricas, 2011), therefore 125 nM GnRH was selected for the target concentration throughout the study. Trials in the outer and inner nuclear layers began with 120 seconds of Ringer's, followed by 120 seconds of 125 nM GnRH, 120 seconds of Ringer's, and 120 seconds of 2 μM antide. The ganglion cell layers were imaged without access to antide, so trials were as follows: 120 seconds Ringer's, 120 seconds 125 nM GnRH, and 60 seconds Ringer's.

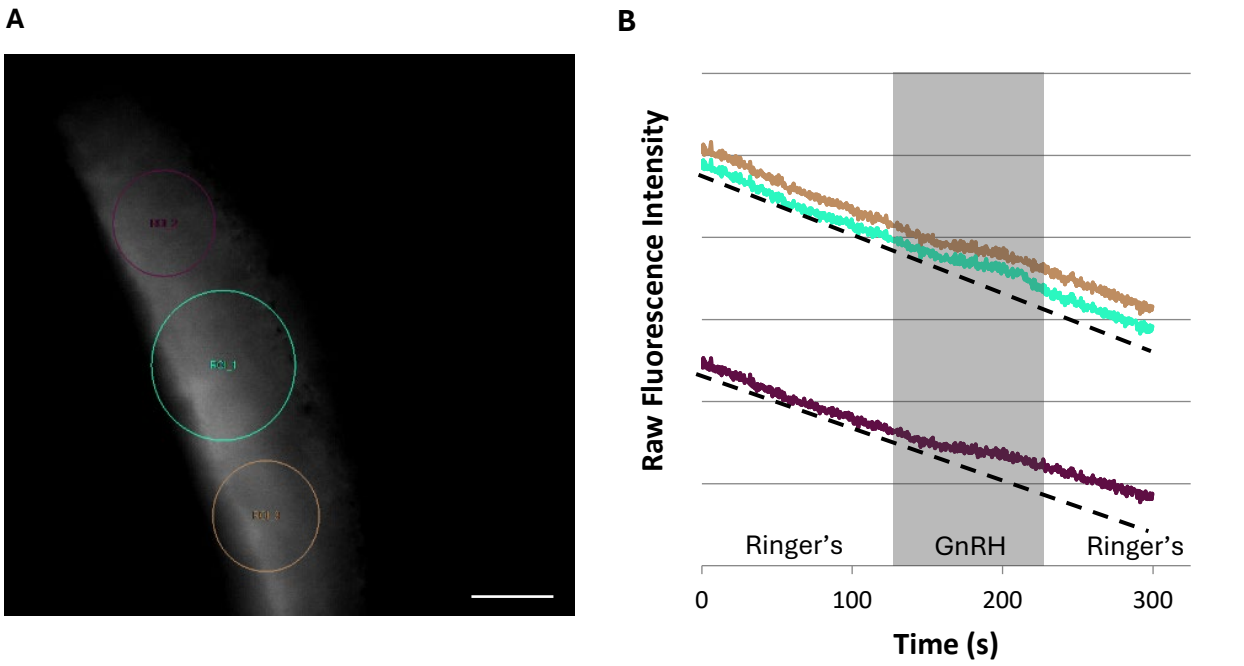


Figure 2. Pilot study showing 125 nm GnRH bath application affected retinal slice calcium activity in ovulated female. (A) Three regions of interest (ROI-1:green, ROI-2:purple, ROI-3:brown) all include some of the ganglion cell and inner plexiform regions. Mean fluorescence of the regions was measured throughout a five-minute recording and are shown without any normalizing or modification (B). GnRH application increased fluorescence in the regions of interest. The first 1-2 minutes of calcium imaging data here and throughout this study always begins with Ringer's as a control to calculate linear fluorescent decay for each individual recording. A linear regression was calculated from the initial decay (illustrated by the dotted line) whose slope was used to normalize the raw fluorescence in the following experiments. Scale bar is 100 μm.

Recordings were analyzed in ImageJ (Schneider, 2012). Retinal layers were outlined, and mean fluorescence of the layer was measured for each image throughout the whole trial. Early recordings with just Ringer's showed the fluorescent indicator, Fluo-4, decayed linearly within timelines of our experiments. Therefore, to adjust for fluorescent decay in the data, a linear regression of the raw fluorescence values during the first Ringer's bath for each trial was used to measure the rate of fluorescent decay, which the recording was first normalized to (dotted lines, Figure 2) before calculating change in fluorescence with $\Delta F = \frac{(F_t - F_0)}{F_0}$.

**Reproductive behavior trials with ovulated female eye injections of saline, GnRH, or antide**

To examine the reproductive behavioral effects of modulated retinal GnRH activity in ovulated females, female eyes were injected with saline, GnRH, or antide shortly prior to a reproductive encounter and behaviors of the fish were quantified. Two nights before the trial, a dominant male was selected from the community tanks and placed, isolated, in the behavior trial tank to provide time for territory establishment. To reduce male stress during this isolated period, the neighboring tank was filled with 2 males and 3 females to allow for visual communication between tanks. One hour after waking on the morning of the trial, an ovulated female was selected from the community tanks. Eye diameter was measured with calipers to estimate total eye volume. From this, injection concentrations were calculated to match final hormone concentrations in the eye with the concentrations used in the calcium imaging experiments (125 nM GnRH and 2 μM antide). The fish were anesthetized in ice cold cichlid water, a small incision was made with a feather scalpel blade (3M), and both eyes were injected with 1 μL of either sham 0.9% saline solution, GnRH, or antide using a blunt-tipped syringe. Eyes were always injected at the same location on the temporal side (closest to the gills) to best avoid the highly complex system of connected ligaments in *A. burtoni* eyes (Khorramshahi et al., 2008). The injection sites were sealed with vetbond (3M), and the female was given 1 hour of recovery in an isolated fish tank.

Following the recovery period, a video recorder was set up facing the isolated male's behavior tank about 3 feet away and a barrier was placed to block the behavior tank from viewing fish in adjacent tanks. The female was placed into the behavior tank and with both fish in frame, they were video recorded for 30 minutes for later analysis.

To ensure consistent reproductive willingness and engagement by males for all trials, the 30-minute timer was started once the male performed 3 courtship behaviors within a 10 second span. If no male courtship was observed within 15 minutes, the trial was terminated and not used for analysis.

**Behavioral analysis of reproductive trials**

Male courtship and female responses to the courtship were quantified using Behavioral Observation Research Interactive Software (BORIS) (Friard & Gamba, 2016). Male courtship behaviors that were quantified included quivers, waggles, and leads which typically occur in a quick, sequential manner. Since females typically respond to these courtship attempts at the end of the sequence, one “behavior group” was defined as sequential male behaviors occurring within 3 seconds, providing a better account of total courtship bouts that females could respond to. The number of behavior groups were used to calculate the percentage of female positive, negative, and non-responses to courtship attempts:

$$\%\ \text{of courtship attempts responded to} = \frac{\#\ \text{of female responses}}{\#\ \text{of male behaviors groups}} \text{x } 100$$

**Neural activation pS6 labeling in retina of female behavior trial participants**

To determine which layers of the female retina were activated during the behavior trials and further characterize GnRH-modulated neural activation changes, females were collected after the behavior trials and their retinas were labeled for the neural activation marker pS6. Collected females were anesthetized and sacrificed with methods described above. The eyes were removed from the head and the lenses were gently removed from the eye through a corneal incision. The eyes were fixed in 4% paraformaldehyde (PFA, Sigma) solution for 24 hours, rinsed in 1x phosphate buffered

saline (PBS, VWR) for 24 hours, and transferred to a 30% sucrose solution (sucrose dissolved in 1xPBS at a final proportion of 30% sucrose by weight) for 24 hours before cryosectioning. The eyes were mounted in OCT media (Tissue-Tek), cryosectioned at 20 µm, collected onto alternate (for redundancy) charged microscopy slides, allowed to dry for 24 hours, and frozen at -80°C until labeling.

Upon neural activation, the S6 ribosomal protein is phosphorylated by internal signaling cascades, resulting in increased translation levels (Knight et al., 2012). The retina sections were labeled for pS6 as previously described and validated (Butler et al., 2019). First, the -80°C microscopy slides with mounted, sectioned retina were thawed to room temperature and a hydrophobic barrier was drawn on the edge of the slide around the tissue (Immedge pen, Vector Labs) and allowed to dry for at least 20 minutes. Slides were washed with 1xPBS (3x10min), and blocked with blocking solution (10 mL 1xPBS, 0.02g bovine serum albumin (Sigma), 30 µL Triton-X-100 (Sigma, high purity), and 500 µL normal goat serum (10%)) for 2 hours at room temperature. Then incubated with the primary pS6 antibody overnight (pS6 ribosomal protein (S235/236) rabbit mAb, Cell Signaling 4858s, 1:1500 final dilution concentration in blocking solution) at 4˚C in the dark in a sealed, humidified chamber. The next day, the slides were washed with 1xPBS (3x10min) and incubated for 2 hours with the secondary antibody (goat anti-rabbit, biotinylated, Vector Laboratories, dilution 1:200 in 1xPBS and 2% normal goat serum). Slides were rinsed with 1xPBS (3x10min), endogenous peroxidases were quenched with 1.5% $H_2O_2$ (VWR) (1x10min), rinsed with 1xPBS (3x10min), and incubated with an avidin-biotin-horseradish peroxidase complex (ABC Elite Kit, Vector Laboratories) for 2 hours at room temperature. They were rinsed with 1xPBS (3x10min) and

diaminobenzidine (DAB) chromogen substrate kit with nickel chloride intensification (Vector Laboratories) for 3 minutes developed the tissue. The slides were rinsed in distilled water, dehydrated in an ethanol series (50% 1min, 70% 1min, 95% 1min, 100% 2x2min) and cleared in xylene (VWR) (2x3min). Slides were cover slipped with cytoseal-60 (Richard Allen Scientific) and allowed to dry flat overnight under the hood.

**Tyrosine hydroxylase labeling in ovulated female retina**

Because GnRH3 axons from the brain terminate near dopaminergic interplexiform cells in the retina, researchers have hypothesized GnRH signaling may affect dopaminergic signaling. To roughly understand the location and patterning of dopaminergic cells in *A. burtoni* retina, ovulated female retina were labeled for the rate-limiting enzyme of dopamine synthesis, tyrosine hydroxylase, which is known to be specific to dopaminergic interplexiform cells.

From the ovulated females used in the calcium imaging experiment, one eye was used for calcium imaging and the other was labeled for tyrosine hydroxylase. The eye was fixed and sectioned as described in the pS6 neural activation section. Labeling for tyrosine hydroxylase was like pS6 labeling except for the following: The primary antibody was anti-tyrosine hydroxylase (Millipore #AB152) with an adjusted 1xPBS amount for a final dilution of 1:300. The secondary antibody used was the alexa-fluor 488 anti-rabbit (Invitrogen, #A-11008) with an adjusted 1xPBS for a final dilution of 1:500. Following the secondary antibody application, the remaining steps took place in the dark. Slides were incubated at room temperature for 2 hours, washed with 1xPBS (3x10min), washed with DI water (1x10min), and coverslipped with DAPI fluorogel (nucleic acid stain, Electron Microscopy Sciences).

**Quantification of pS6 neural activation**

To analyze neural activation in different retinal layers of ovulated females who received eye-injections, the number of pS6-labeled cells were quantified across different layers. Labeled tissue was examined on a Nikon Eclipse microscope (20x objective) with Nikon Elements software and photographs were taken with a scope-mounted digital camera. Like calcium imaging, all quantification was done near the optic disc to maintain a consistent quantification location between animals. A 500 μm curved line was drawn starting at 100 μm away from the optic disc and the number of pS6-labeled cells was quantified for the inner nuclear layer (outer 2/3 of inner nuclear layer), amacrine cell layer (inner 1/3 of inner nuclear layer), and the ganglion cell layer. Cell number was calculated as the mean number of labeled cells in the quantified layer across three consecutive sections.

**Statistical analysis**

No statistical analysis was performed on the calcium imaging data due to low sample numbers. The behavior and retina neural activation data were analyzed using SigmaPlot 12.3 (Systat Inc., San Jose, CA, USA). Differences between sham, GnRH, and antide spawning rates were compared using a chi-square analysis where the groups were the eye injection treatments and the conditions were whether spawning occurred or not. Differences between sham, GnRH, and antide group behaviors were compared using one-way ANOVAs with post-hoc Tukey tests. Grubb's test was used to test for outliers, which were excluded from the one-way ANOVA tests and the plots. If the raw data did not meet normality, square root and log transformations were performed to meet normality. If normality could not be met, a Mann-Whitney U-test was

performed. The statistical significance of two-tailed p=0.050 was standard across all analyses.

# RESULTS

## Calcium imaging of live retinal tissue from ovulated females in response to GnRH or antide

To examine the GnRH-modulated activity of different retinal cells of ovulated female *A. burtoni*, living retinal slices were loaded with the fluorescent calcium indicator, Fluo-4 AM, and individual retinal layers were imaged while bathing the whole slice in 125nM GnRH or 2μM antide (GnRHR antagonist) for 2-3 minutes each (Figure 3). One slice was imaged for each recorded retinal layer using 2 different animals. Fluo-4 fluorescence was broadly observed in all cell layers. Immediately prior to GnRH treatments, bathing in Ringer's solution (sham treatment) while imaging revealed the linear photobleaching to which all data from each individual trial were normalized. GnRH application decreased mean fluorescence in the outer nuclear layer within 15-30 seconds. This effect often lasted the remainder of the trial, for 2-5 minutes persisting in the wash. The amacrine cell layer at the border of the inner nuclear and plexiform layers, and the ganglion cell layer both increased in fluorescence with GnRH application. Across all tested layers, the antide treatment that followed GnRH had little effect on the fluorescence trends. These data show that 125 nM GnRH treatment impacts layer-specific calcium signaling in live retinal tissue.

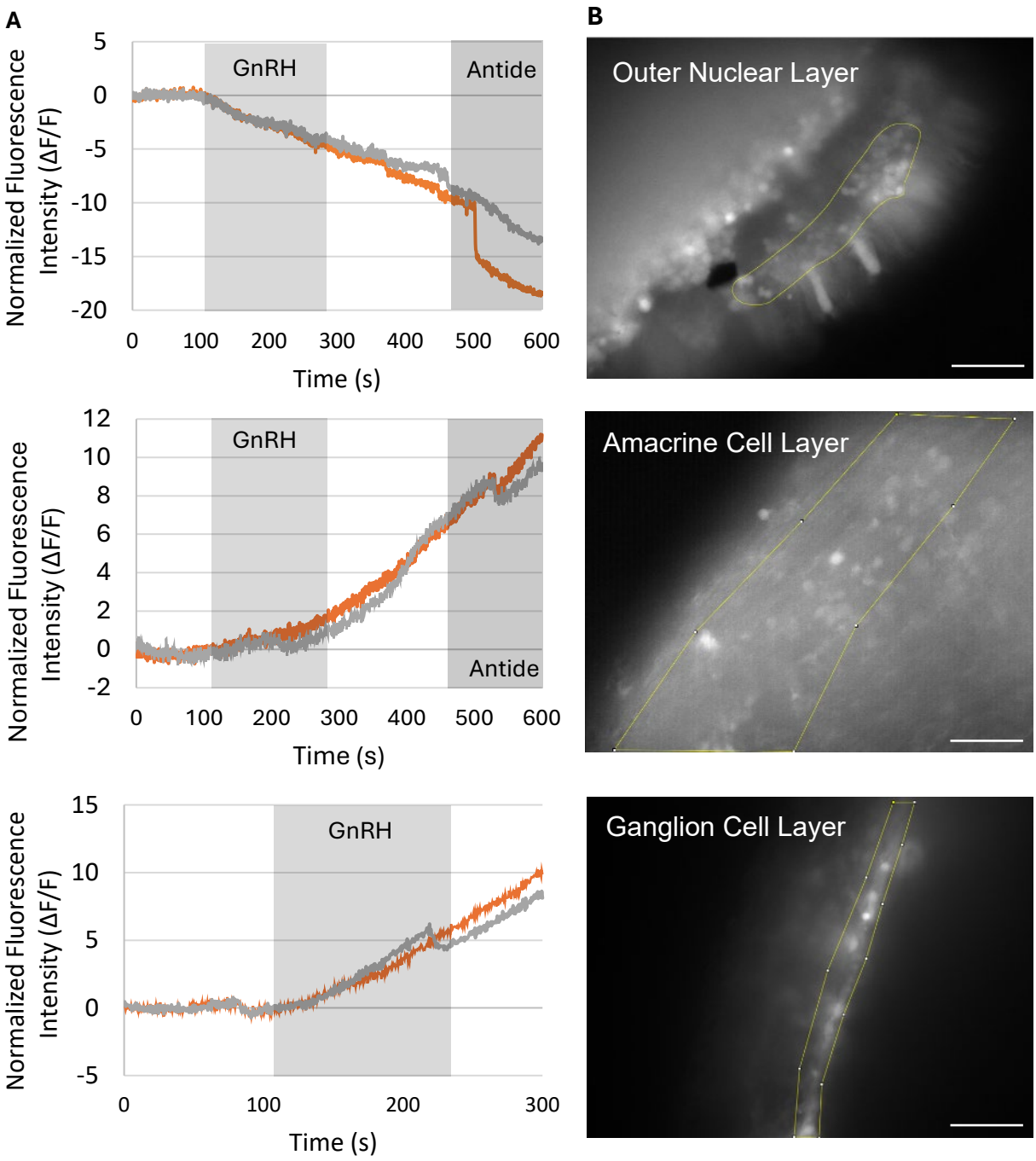


Figure 3. GnRH increases calcium fluorescence in the ganglion and amacrine cell layer but decreases fluorescence in the outer nuclear layer. Fluorescence intensity plots (A) were normalized as described in the methods. The orange line represents a single recording of the mean fluorescence of a whole cell layer (within the imaging frame, (B)), and the gray lines represent a single representative cell of a different live retinal slice. Scale bar = 30 μm.

**GnRH and antide effects on reproductive behavior**

To test the reproductive behavioral effects of GnRH and antide, ovulated females with saline (sham), GnRH, or antide eye injections were placed in an isolated tank with a male for 30 minutes after a short period of recovery. No difference in spawning rates were observed between trials with sham, GnRH, and antide-treated females (Figure 4) (chi-square test, p=0.8418; trials where spawning occurred: sham-treated 66% (4/6), GnRH-treated 57% (4/7), antide-treated 50% (3/6)).

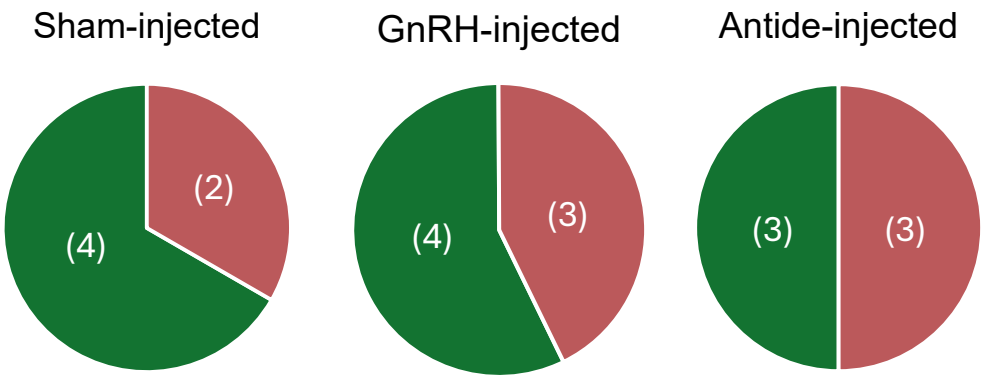


Figure 4. Altered GnRH activity in the retina of ovulated females did not affect the likelihood of spawning. Numbers in parentheses represent sample sizes.

Male behavior was quantified to determine any differences in courtship between the trials (Figure 5). This simultaneously ensured a minimum amount of courtship was present in each trial and could suggest any reciprocal treatment-mediated effects that female responses had on further male courtship within trials. The most common male courtship behaviors (quiver, waggle, lead) were not different depending on female eye injections (quiver: F=0.200, df=2, p=0.821; waggle: F=0.503, df=2, p=0.614; lead: F=0.516, df=2, p=0.606). Also, since these go-to courtship behaviors commonly occur in quick succession, the total number of courtship attempts, called behavior groups, were

quantified and showed no difference across female injection treatments (F=0.194, df=2, p=0.825).

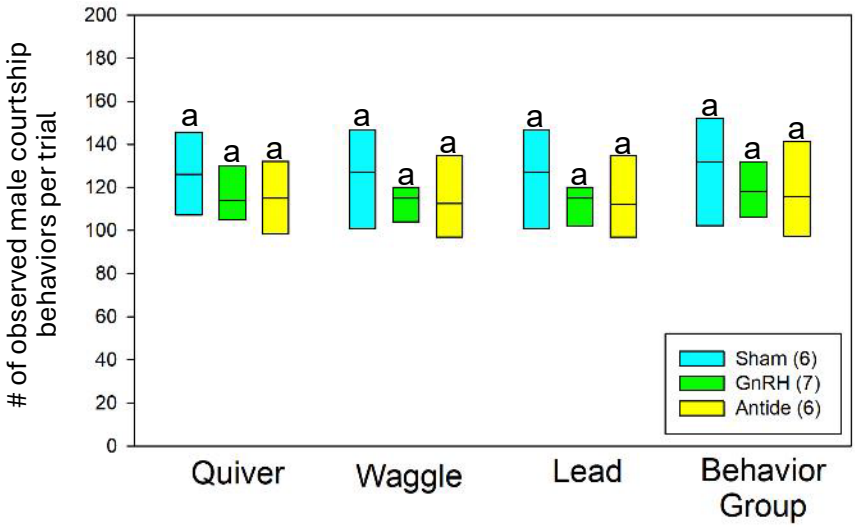


Figure 5. Sham, GnRH, and antide female eye injections did not affect male courtship behavior. The total number of common male courtship behaviors which typically occur in quick succession (quiver, waggle, lead) and the total courtship attempts (behavior groups) measured across the 30-minute behavior trials. Lower box plot boundary=25$^{th}$ percentile; upper box plot boundary=75$^{th}$ percentile; solid line in box=median; dashed line in box=mean; different letters=p-value<0.05; numbers inside legend=sample sizes.

With all trials combined, the percentage of courtship attempts that females responded to, whether the response was to follow or avoid (total responsiveness), was similar in sham and GnRH trials, but lower in antide-injected females than sham (H=6.457, df=2, p=0.040, Post-hoc (Dunn's Method: sham-GnRH: q=1.768, sham-antide: q=2.459, GnRH-antide: q=0.864) (Figure 6, left). Of those total responses, female eye injections did not affect the percentage of follows or avoidance responses to courtship attempts across all trials (follow: H=1.872, df=2, p=0.392, avoid: H=0.818, df=2, p=0.664) (Figure 6, right).

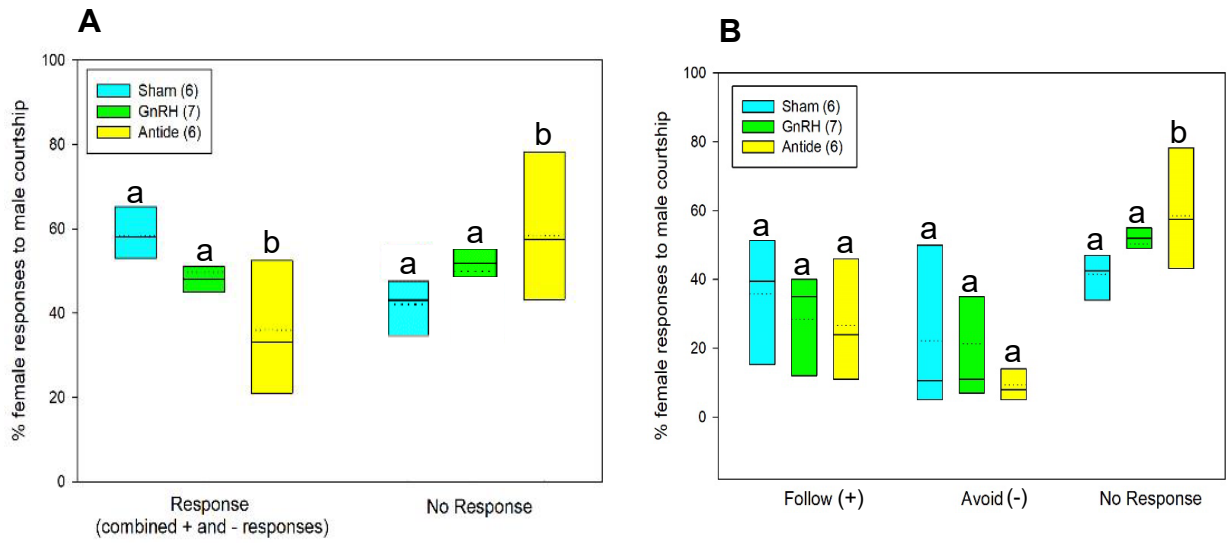


Figure 6. From data across all trials, antide eye injections reduced overall female responsiveness to male courtship. However, GnRH and antide did not affect whether the response was positive or negative. Shown are a plot with combined responsiveness data (left) and a plot with the combined responsiveness split into positive (follow) or negative (avoid) (right). Female follows were counted if the female turned her body towards the courting male or the spawning territory within 3 seconds of a courtship attempt. Avoids were counted if the female turned her body away within 3 seconds. Non-responses were when the female neither followed nor avoided. Percentages were calculated using the ratio of the number of female responses to the number of male behavior groups (courtship attempts) throughout a 30-minute trial. Lower box plot boundary=25$^{th}$ percentile; upper box plot boundary=75$^{th}$ percentile; solid line in box=median; dashed line in box=mean; different letters=p-value<0.05; numbers inside legend=sample sizes.

While the female eye injections had little impact on the average behavior across all trials combined, there was a large distribution among follow responses within treatments, and a notable difference in distribution among the avoidance and non-responses in GnRH- and antide-injected females, respectively. Accounting for whether spawning occurred, in trials where spawning did *not* occur, GnRH-injected females were more likely to actively avoid male courtship behaviors than antide-injected (t=2.809, df=4, p=0.024) whereas antide-injected females had less active avoidance and were more likely to not respond to courtship at all (t=3.674, df=4, p=0.011) (Figure 7). In trials where spawning did occur, no differences between total responsiveness (F=4.040, df=2,

p=0.068) or types of responsiveness (follow: F= 0.988, df=2, p=0.414, avoid: F=0.529, df=2, p=0.609) were observed. Collectively these data show that in our behavior test setup, female GnRH and antide eye injections had little effect when the pair ultimately decided to spawn. Instead, GnRH and antide eye injections mostly impacted female decision-making and response to courtship when spawning did not occur.

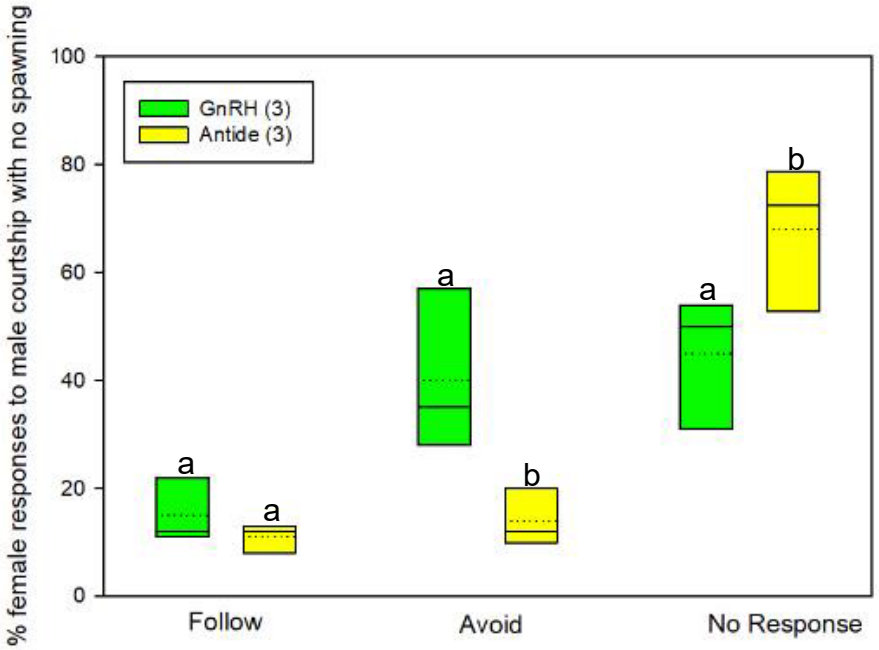


Figure 7. Across only trials where spawning did not occur, females with GnRH eye injections often chose to actively avoid courtship, whereas antide-injected females were more likely to not respond at all. Female follows were counted if the female turned her body towards the courting male or the spawning territory within 3 seconds of a courtship attempt. Avoids were counted if the female turned her body away within 3 seconds. Non-responses were when the female neither followed nor avoided. Percentages were calculated using the ratio of the number of female responses to the number of male behavior groups (male courtship attempts) throughout a 30-minute trial. Lower box plot boundary=25$^{th}$ percentile; upper box plot boundary=75$^{th}$ percentile; solid line in box=median; dashed line in box=mean; different letters=p-value<0.05; numbers inside legend=sample sizes.

**GnRH and antide effects on pS6-labeled neural activation in the retina**

To determine which cells were affected by the experimental GnRH modulations, ovulated female retinas were collected, sectioned, and labeled for the neural activation marker, pS6 (phosphorylated ribosome that corresponds with cellular activation within the last ~1 hour), after the behavior trials. Positive pS6 labeling was observed and quantified in the inner nuclear and ganglion cell layer of all females (Figure 8). GnRH-injected females had the highest number of pS6-labeled cells in the ganglion cell layer ($F=25.473$, $df=2$, $p=0.007$) and the inner nuclear layer ($F=38.020$, $df=2$, $p=0.004$), about twice as many compared to sham and antide-treated females (Figure 9). Further, within the inner nuclear layer, the most dense labeling occurred in the amacrine cell layer ($F=52.155$, $df=2$, $p=0.002$), which also had more pS6-labeled cells than both sham- and antide-treated females. Sham- and antide-treated ovulated females showed no differences in retinal pS6 labeling within all quantified layers.

**Tyrosine hydroxylase labeling in *A. burtoni* retina**

Based on conclusions from other studies, GnRH may act through the dopaminergic system in the retina. While not confirmed, the pattern of cells with the most dense pS6-labeling in the amacrine cell layers of ovulated females (Figure 8) resembles the layout of dopaminergic interplexiform cells in the retina (Figure 10). Therefore, the activity of dopaminergic interplexiform cells during the behavior trials may have been affected by the GnRH treatment.

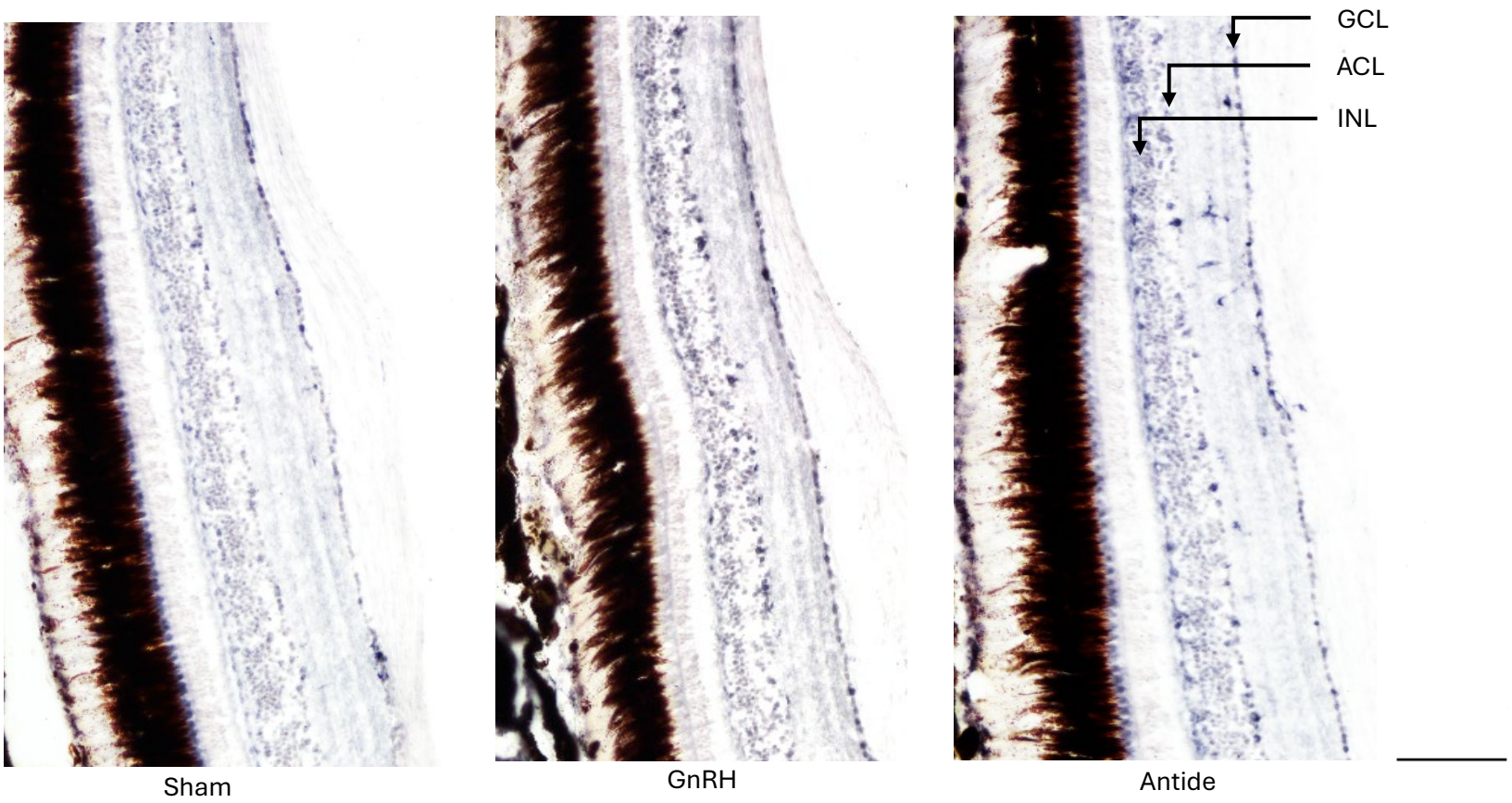


Figure 8. Representative pS6 labeling of sham, GnRH, and antide-injected retina of females from the behavior trials. Some background staining is visible. Dark cells are labeled with pS6 and were confirmed under the scope in real time during quantification.
GCL = ganglion cell layer.
ACL = amacrine cell layer (inner 1/3 of the inner nuclear layer).
INL = inner nuclear layer (outer 2/3 of the inner nuclear layer).
Scale bar = 100 μm.

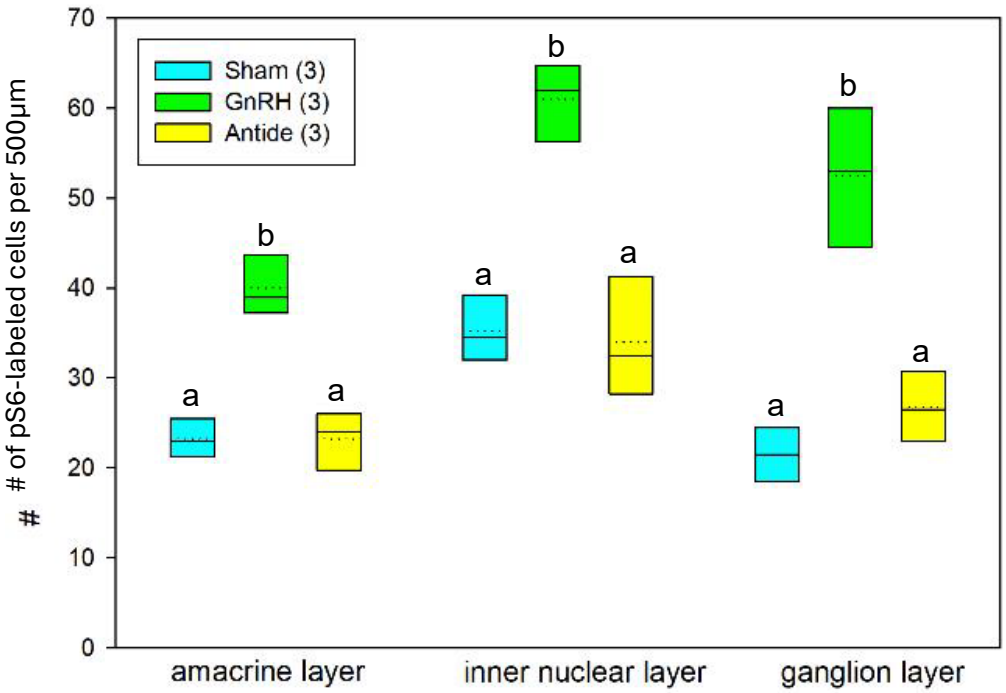


Figure 9. GnRH-injected females from the behavior trials had more neural activation in the amacrine, inner nuclear, and ganglion cell layers than sham or antide-injected females. Total pS6-labeled cells along a 500µm length were counted per layer and compared between injection treatments. Lower box plot boundary=25$^{th}$ percentile; upper box plot boundary=75$^{th}$ percentile; solid line in box=median; dashed line in box=mean; different letters=p-value<0.05; numbers inside legend=sample sizes.

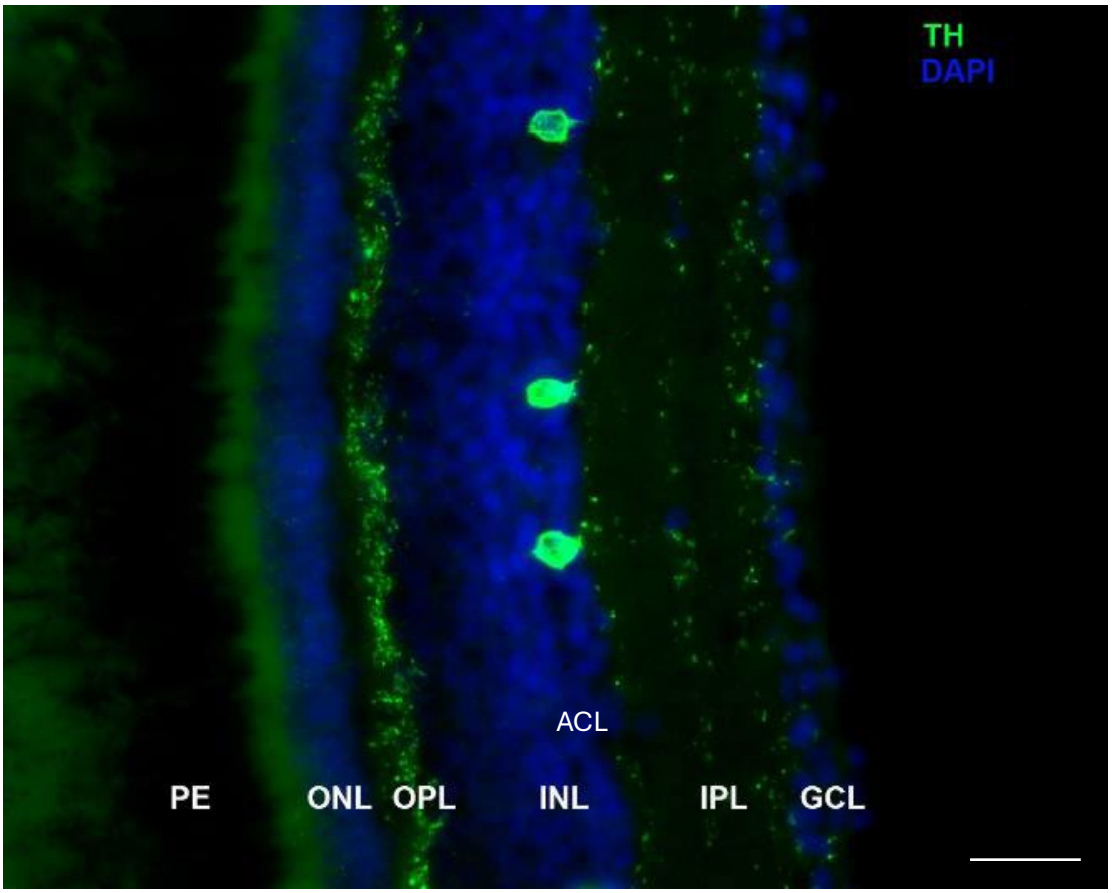


Figure 10. Dopaminergic interplexiform amacrine cells in *A. burtoni* ovulated female retina. Immunohistochemistry label for tyrosine hydroxylase (TH, green) labels interplexiform cell bodies and fibers. Total cell bodies are visualized with the fluorescent nucleic acid stain, DAPI. PE = pigmented epithelium. ONL = outer nuclear layer. OPL = outer plexiform layer. INL = inner nuclear layer. ACL = amacrine cell layer. IPL = inner plexiform layer. GCL = ganglion cell layer. Scale bar = 50 μm.

# DISCUSSION

Here, we illustrate GnRH-mediated activation changes in the retina and reproductive behavior of ovulated female *A. burtoni*. Using fluorescent calcium-imaging in live retinal tissue, we show that GnRH affected calcium signaling across major nuclear layers in the retina, with opposing effects between the outer nuclear layer, and the inner and ganglion cell layers. We also show that eye injections of GnRH in ovulated females increased avoidance behavior responses to male courtship in trials where spawning ultimately did not occur. Further, antide eye injections resulted in less spawning compared to sham and GnRH, and decreased the overall responsiveness of females to male courtship across all trials and when spawning did not occur. Finally, pS6 neural activation immunohistochemistry of retina from females participating in the behavior trials showed no difference in pS6-positive cell counts between sham and antide-injected females in the whole inner nuclear, amacrine cell, or ganglion cell layers. However, GnRH-injected females had increased pS6-positive cells in the inner nuclear, amacrine cell, and ganglion cell layers. Collectively, this study provides early find a better word than early evidence that GnRH can modulate activity of multiple cellular regions across the retina in ovulated female *A. burtoni*, impacting reproductive visual processing in the retina, decision-making, and success.

### GnRH neurons, GnRH receptors, and GnRH activity observed with calcium imaging

The existence of GnRH neurons in the brain whose axons travel along the optic nerve and innervate the retina of teleosts have long been known (Stell et al., 1984; Zucker & Dowling, 1987), but few studies have investigated the action of GnRH in the retina. The bulk of GnRH axons branch fibers at the border of the inner nuclear and

inner plexiform layers. Further, GnRH fibers mostly weave around dopaminergic interplexiform amacrine cells in a “basket-like” manner in coral reef fishes, goldfish, and others (Maruska & Tricas, 2007; Stell et al., 1984). With all this seemingly related structural evidence connecting GnRH fibers with the amacrine cell layer, it was initially thought that GnRH acts solely or mostly within this layer, likely inducing downstream action of dopamine on other cells within the inner nuclear layer (Maruska & Tricas, 2007; Umino & Dowling, 1991). In this study, we show that GnRH modulates calcium signaling in the amacrine cell layer, further supporting the hypothesis that cells within this layer play a role in the GnRH-mediated visual plasticity. However, more recently, *A. burtoni* GnRHRs were found to exist throughout all retinal nuclear layers (Grens et al., 2005). The data in this study showing modulated calcium fluorescence in the ganglion cell layer and outer nuclear layer provides evidence that GnRH affects activity of other layers besides the inner nuclear layer. Lastly, the highly branched but localized GnRH axons from the terminal nerve ganglia are electrically coupled for synchronized firing (Haneda & Oka, 2008) and have large vesicles (Zucker & Dowling, 1987), which shows structural similarity to GnRH neurons innervating reproductive-related brain regions (Moore et al., 2018). The conclusions from within and outside of this study collectively suggest an efficient mechanism for regulating visual plasticity through large releases of GnRH that may act in a paracrine fashion across the whole retina. Overall, these data support the broadened scope of GnRH’s direct effects within the retina beyond acting on only dopaminergic amacrine cells.

**Potential calcium-mediated mechanisms in this study**

In neurons, signals carried by changes in intracellular calcium are important for neuronal electrical properties through voltage-gated calcium channels, and signal

transduction pathways ultimately affecting discrete synaptic activity, synaptic plasticity, gene expression, and more (Grienberger & Konnerth, 2012). Retinal neurons have long served as a popular model to study calcium's role in intracellular signal transduction pathways across a variety of receptor types including GnRHRs. GnRHRs are membrane-bound G protein-coupled receptors, and within cells expressing GnRHRs, receptor activation causes sequential activation of G proteins, phospholipases, and second messengers to release calcium from intracellular storage to ultimately affect visual signal processing. Because of the broad role of calcium in the retina, we cannot rule out that some observed effects were not due to downstream horizontal or vertical processing of the light-induced activation from imaging and the GnRH treatment. Early electrophysiological evidence using white perch showed GnRH perfusion in flat-mounted whole retina can depolarize horizontal cells (Umino & Dowling, 1991). However, these effects were blocked with haloperidol, a dopamine receptor blocker, suggesting GnRH acted through the dopaminergic system to change horizontal cell activity. The modulated horizontal cell activity took >1 minute for observed responses, suggesting downstream effects of GnRH on horizontal and vertical processing may take longer than 1 minute. Our study showed early GnRH responses in all imaged layers were much more rapid, occurring in as little as 15 seconds. The horizontal cell study used whole retina instead of sliced retina, so the amount of GnRH reaching the different retinal layers could have differed compared to this study. However, they intentionally perfused both sides of the retina, so GnRH was likely able to reach all retinal layers to a sufficient degree. Together, these studies suggests that the immediate fluorescence changes following GnRH were potentially a direct cause of GnRH receptor activation on

the imaged cells, and further imaging past ~1 minute within each trial possibly included other horizontal and/or vertical signal processing as well.

Finally, antide was used following the GnRH application in an attempt to reverse the effects of GnRH. Antide produces a very strong and immediate competitive blockage of GnRHRs (Huirne et al., 2004; Tomabal et al., 2017). Further, the concentration used in this study was high enough to block all GnRHR types (Balik et al., 2009; Maruska & Tricas, 2011), yet no reversal effect was observed. It's possible that blocking the receptors did not cease the already-initiated intracellular signal transduction from the activated receptors. Therefore, the downstream changes in signaling processing that were still developing/proceeding from the initial GnRH application were masking the effects of antide. Future calcium imaging with GnRH and antide could use antide before the GnRH treatments instead of after to better illustrate any blocking by antide. Also, antide treatment of ~2 minutes may not have been long enough to see the effects, so future studies should experiment with longer periods of treatment and recording.

**GnRH and antide eye injection effects on reproductive behavior**

Vision is one of the most important senses for reproductive communication across many vertebrates, including cichlids (Maruska & Fernald, 2018). Many animals have diverse coloration and behavioral displays that are critical to their courtship and spawning behaviors. Because female *A. burtoni* invest so much energy into reproductive preparation, and 2 weeks of maternal care when she can't eat, reproductive decisions can have direct consequences on her survival. Retinal GnRH has been suggested to be involved in motivation of male and female sexual behaviors in both fish and rodents (Okuyama et al., 2014; Wirsig & Leonard, 1987; Yamamoto et al.,

1997). Previously, we've shown that female affiliative behaviors with courting males positively correlated with *gnrhr2* mRNA levels in the retina (Butler et al., 2019), suggesting GnRH plays a role in reproductive state-dependent visual plasticity that can ultimately affect behavior in *A. burtoni*. Combined with evidence that ovulated female *A. burtoni* have improved visual sensitivity to a wide range of wavelengths, we hypothesized GnRH signaling to the retina may allow for better detection and assessment of potential mates. This study showed spawning can still occur with exogenous GnRH receptor activation or inhibition in the retina, but reproductive success is decreased with antide eye injections, suggesting antide may reduce the ability to detect or assess mates.

Innate social behaviors in vertebrates have been categorized into four stages: 1) *detect* a potential social target, 2) *approach* the social target, 3) *investigate* to gain more information about a conspecific, and 4) the *consummatory action phase* where the social behavior occurs (Ogawa & Parhar, 2022; Wei et al., 2021). These different phases are associated with different circuitry within the social decision-making network, a highly conserved network of social processing and reward center regions in the brain of vertebrates (O'Connell & Hofmann, 2012). While the male and female fish in this study must have ultimately detected each other to initiate reproductive behaviors, the burden of detection on the visual system is decreased since we intentionally placed two reproductively-ready conspecifics into a small tank with optimal water conditions. In other words, the benefits of improved visual sensitivity in ovulated females may not have a large impact on near-field detection of the colorful male who is in ideal clear and still water conditions. This may explain why we observed fewer behavior differences

across eye injection groups than expected. It's possible that the improved visual sensitivity is most useful in ovulated females when searching for mates at a farther distance in less ideal water conditions as occurs in nature. This hypothesis is driven by the differences in overall responsiveness between GnRH and antide eye-injection treatments, where GnRH-injected females had overall higher total responsiveness (following and avoiding combined) to courtship than antide but did not follow more courtship attempts than sham-treated. This suggests that GnRH may not be directly inducing reproductive behaviors like prostaglandins and other hormones are known to in *A. burtoni* (Juntti et al., 2016) but plays a role in enhancing the processing of reproductive-related visual signals, allowing selective females to make more decisive and informed decisions about spawning. The decreased responsiveness from antide-injected females might suggest they are not detecting the extent of reproductive signals that GnRH-injected females were, so instead of actively following or avoiding courtship attempts, they were more likely to not respond at all.

**GnRH and antide effects on pS6 neural activation and downstream processing**

This study shows that compared to sham and antide injections, GnRH-injected *A. burtoni* females have increased neural activation (increased pS6-labeling) in the amacrine, inner nuclear, and ganglion cell layers. GnRH has the potential to modify neural activation in the retina and ultimately reproductive behavior through several pathways. Aside from the already discussed direct effects of GnRH on retinal cells, retinal GnRH can affect downstream dopaminergic signaling by acting on dopaminergic interplexiform amacrine cells in the retina. It is already known that ovulated *A. burtoni* females have more tyrosine hydroxylase (rate-limiting enzyme for dopamine synthesis) in the eye than any other reproductive stage (Henry, 2023). Further, the dense pS6

labeling of cells in the amacrine cell layer appears similar in layout to the known positions of dopaminergic interplexiform cells (Figure 10). Combined with this study showing increased neural activation in the amacrine cell layer of GnRH-injected females, a GnRH-induced increase in dopaminergic activity in reproductive females is plausible. As one of the most abundant neuromodulators in the retina, dopamine has known roles in visual plasticity through regulating various voltage-gated ion channels (Witkovsky, 2004) and modulating opsin levels in both rods and cones of fish and amphibians (Alfinito & Townes-Anderson, 2001; Li et al., 2005). Because GnRH can indirectly affect *A. burtoni* opsin levels, which are known to change across the reproductive cycle (Butler & Maruska, 2021), GnRH could drive changes in the detection of salient visual signals. Because GnRH effects in the retina can be prevented by a dopamine receptor blocker (Umino & Dowling, 1991) and GnRH neuron fibers are known to surround dopaminergic cells in the retina (Maruska & Tricas, 2007), future studies investigating mechanistic connections between the reproductive cycle, GnRH, and dopaminergic activity in the retina could prove useful.

Dense pS6 labeling was often seen on the outer side of the inner nuclear layer, possibly in horizontal cells. Early investigations into white perch horizontal cells using microelectrode recordings showed several “neuroactive substances” such as FRMFamide, GABA receptor inhibitors, and GnRH can impact horizontal cell activity (Umino & Dowling, 1991). GnRH caused horizontal cells to depolarize, but this response halted when the whole retinal mounts were treated with a dopamine receptor blocker (Umino & Dowling, 1991). This suggests the GnRH-mediated increase in this study, in (presumably) horizontal cell neural activation, may have been operating

through the dopaminergic system. Next, since ganglion cells are the final output neurons of the retina, increased neural activation in the ganglion cell layer of GnRH-injected females can heavily impact central visual processing. Ganglion cells are known to send signals through the optic nerve to the brain and to convey information about the color, shape, and movement of an object. Therefore, ganglion cell activity changes in females of this study may impact processing of a courting dominant male displaying bright coloration patterns, flared fins for a larger appearance, and rapid swimming motions to signal reproductive fitness and health. GnRH from the terminal nerve ganglia has already been associated with modulating ganglion cell processing of color contrast in goldfish (Reperant et al., 2006; Stell et al., 1984), but more evidence is necessary to draw strong conclusions for the effects of processing in the retina and downstream in the brain.

Across all measured retinal layers, antide injections did not affect pS6 labeling compared to sham. While we expected to see opposing effects from GnRH, there are several reasons why they may not have occurred. First, antide was injected into already ovulated females. Some of the biggest changes in reproductive physiology and visual plasticity happen during the transition *into* ovulation (Butler et al., 2019). Therefore, the window to block any large change in GnRH activity may have already passed, so the antide may not have affected much. Also, this treatment was predicted to decrease pS6 in the retina. With the 90 minutes total from time of eye injection to eye dissection, the natural levels of retinal pS6 may not have had enough time to fall even if antide was having an effect. Finally, the concentration of antide was high enough to theoretically block all GnRH receptors, but that assumes antide injected into the vitreous was able to

effectively reach the retina. Based on the strong results in the GnRH-injected pS6 labels, it appears GnRH sufficiently contacted retinal tissue. Further, GnRH and antide are similar in molecular weight so it is likely that antide reached the retina at sufficient levels, but no effect was observed due to the previously stated reasons.

Collectively, these GnRH-mediated mechanisms can affect visual processing in the retina to ultimately affect visual processing in the brain and behavioral output. In teleosts and vertebrates broadly, ganglion cells project not just directly to primary visual processing centers (optic tectum in teleosts) (Presson & Fernald, 1986), but also to the preoptic area, ventral and dorsal thalamus, hypothalamus, and more (Baier & Wullimann, 2021; Fernald, 1982; Northcutt & Butler, 1993). These brain regions can impact both reproductive physiology and reproductive behavior. Projections to the preoptic area connect to magnocellular and parvocellular preoptic nuclei and the suprachiasmatic nucleus (Presson et al., 1985). One way the preoptic nucleus can affect reproduction is through the neuropeptide arginine vasotocin, which is synthesized and release by both magnocellular and parvocellular neurons (Foran & Bass, 1998). Vasotocin and the mammalian homolog vasopressin have been shown important for reproductive regulation and behavior in many animals (Caldwell, 2017) including fish (Butler et al., 2021), amphibians (Diakow & Nemiroff, 1981), reptiles (Wilczynski et al., 2017), and mammals (Wang et al., 1998). Next, the suprachiasmatic nucleus has known roles in circadian rhythm and coordinates neuroendocrine events to maximize reproductive success (Williams & Kriegsfeld, 2012). Lastly, dorsal and ventral thalamic nuclei process and relay reproductive information (Fernald, 1982; Usrey & Alitto, 2015). For example, unilateral optic nerve transections in midshipman fish decreases thalamic

GnRH expression (Foran et al., 1997). Together, these are downstream mechanisms which GnRH-altered retinal activity may impact, leading to changes in reproductive physiology and behavior.

**Conclusions**

My study investigated the role of GnRH in mediating reproductive state-dependent visual plasticity in ovulated female *Astatotilapia burtoni*. Using two different measures of neural activity, I showed that retinal GnRH in ovulated females impacts activity of cells in multiple retinal layers including the outer, inner, amacrine, and ganglion cell layers. I also showed that GnRH eye injections don't increase the likelihood of spawning under already ideal spawning conditions, but potentially allow females to make more informed decisions, shown by increased avoidance compared to sham and antide when she decides against spawning. Ovulated females have increased visual sensitivity, and these data collectively inform us on mechanisms that may be responsible for this visual plasticity. The strong increase in amacrine cell layer intracellular calcium and pS6 labeling in response to GnRH support the working theory that GnRH signals to dopaminergic interplexiform amacrine cells (Grens et al., 2005; Umino & Dowling, 1991), which are major effector of retinal plasticity. Further, my project expands on this understanding by illustrating that GnRH likely has direct effects on other cell types throughout the retina.

In the path to understanding how vertebrates tune sensory systems to their internal and external conditions, this study lays foundation for future studies to build on. For example, a future study could investigate why differences in behavior across treatments were more subtle than expected. I predict this may have been due to the ideal spawning conditions used in this study – little external pressure to spawn, clear

water, extremely dominant, colorful, and visible males and spawning territories, being well-fed, etc. It is likely that *A. burtoni* experience turbid water environments in Lake Tanganyika due to water turbulence and animal activity, such as elephants and hippos running through the shallow waters where these cichlids often reside. Increased water turbidity, for example, could be used to reduce these ideal spawning conditions to provide more headroom for observed behavioral changes. Further, this study only used ovulated females because this stage is when dramatic increases in visual sensitivity occur. This proved to be a limitation of the study as mechanisms responsible for this plasticity may have already happened closer to the onset of ovulation. Therefore, a more effective use of antide could be a several-day treatment in the eye as the fish nears ovulation to prevent any GnRH signaling from occurring. Following that treatment with the same pS6 labeling and even using females across other reproductive stages may provide more useful information towards how GnRH operates. Lastly, while GnRH is positioned to be a key player in reproductive state-dependent visual plasticity with its anatomical and physiological connections to both the reproductive cycle and the retina, there are other peptides to study. For example, androgen receptors (*arα*, *arβ*), estrogen receptor (*erα*), progesterone receptor (*pgr*), luteinizing hormone receptor (*lhr*), and dopamine receptors (*drd1a, drd2a*) all have regulated expression across the reproductive cycle in *burtoni* female retina (Butler et al., 2019; Henry, 2023) and may affect reproductive state-related visual sensitivity.

In conclusion, this project is the first to combine behavioral and physiological experiments to understand mechanisms of reproductive state-dependent visual plasticity. Because *burtoni* females spend weeks preparing for reproduction and do not

eat for 2 weeks after spawning, reproductive decisions have dramatic consequences on both mother and fry survival. This study lays the foundation for future studies to expand on vertebrate reproductive communication and visual plasticity, with far-reaching consequences on an animal's ability to meet its internal and external environmental demands to increase species survival.

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# VITA

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