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Reproductive state-dependent plasticity in the visual system of an African cichlid fish

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ABSTRACT

Visual communication is used widely across the animal kingdom to convey crucial information about an animals' identity, reproductive status, and sex. Although it is well-demonstrated that auditory and olfactory sensitivity can change with reproductive state, fewer studies have tested for plasticity in the visual system, a surprising detail since courtship and mate choice behaviors in many species are largely dependent on visual signals. Here, we tested for reproductive state-dependent plasticity in the eye of the cichlid fish *Astatotilapia burtoni* using behavioral, gene expression, neural activation, and electrophysiology techniques. Males court ovulated females more intensely than gravid females, and ovulated females were more responsive to male courtship behaviors than gravid females. Using electroretinography to measure visual sensitivity in dark-adapted fish, we revealed that gravid, reproductively-ready females have increased visual sensitivity at wavelengths associated with male courtship coloration compared to non-gravid females. After ovulation was hormonally induced, female's spectral sensitivity further increased compared to pre-injection measurements. This increased sensitivity after hormone injection was absent in non-gravid females and in males, suggesting an ovulation-triggered increase in visual sensitivity. Ovulated females had higher mRNA expression levels of reproductive neuromodulatory receptors (sex-steroids; gonadotropins) in the eye than nonovulated females, whereas males had similar expression levels independent of reproductive/social state. In addition, female mate choice-like behaviors positively correlated with expression of gonadotropin system receptors in the eye. Collectively, these data provide crucial evidence linking endocrine modulation of visual plasticity to mate choice behaviors in females.

1. Introduction

Visual communication is used widely in invertebrates and vertebrates to convey crucial information about an animals' identity, motivation, reproductive status, and sex. During reproduction, animals often increase their use of visual displays like courtship dances or changes in coloration (Osorio and Vorobyev, 2008). In most animals, males are the senders of these reproductive signals while female receivers use this information for mate choice. Coloration and ornament size can also be an indicator of parasite load and overall health, which provides additional mate choice information (Houde and Torio, 1992; Molnár et al., 2013; Ness and Foster, 1999; Thompson et al., 1997). For animals that use visual social displays, the ability to optimally detect these signals is of extreme importance, especially for species that are seasonal breeders and those that cycle in and out of receptive reproductive condition.

Reproductive state is known to modulate sensory detection and perception, both at peripheral structures and central processing regions, such that animals that are ready to reproduce are often better able to detect signals from the opposite sex. Specifically, in fishes, amphibians, and birds, receptive females are better able to detect their mates' calls and/or have an increased response to male calls (Caras et al., 2010; Lynch and Wilczynski, 2008; Maney and Pinaud, 2011; Maruska and Sisneros, 2015; Maruska et al., 2012; Miranda and Wilczynski, 2009; Sisneros and Bass, 2003), and chemosensory capabilities are known to change with both reproductive and metabolic state across taxa (Mousley et al., 2006; Nikonov et al., 2017; Palouzier-Paulignan et al., 2012). Because reproductive-state plasticity exists in peripheral auditory and olfactory structures (i.e. ear, olfactory epithelium), it is likely that similar changes occur in the retina. However, evidence for functional plasticity in the peripheral visual system is limited across taxa.

Reproductive molecules, like sex steroids, are widely studied for their role in modulating sensory function, including vision. In

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mammals, for example, estrogens are essential for healthy ocular function (Affinito et al., 2003). Decreased estrogen signaling after menopause is linked to decreased tear production (Mathers et al., 1998), and estrogen signaling may help protect against age-related eye diseases (e.g. glaucoma) (Vajaranant et al., 2010; Zhou et al., 2007). Estrogens may even be produced in the eye (Cascio et al., 2007), androgens were shown to affect visual sensitivity in male fishes (Shao et al., 2014; Yue et al., 2018), and exogenous estrogens influence opsin expression in the eye of mosquito fish (Friesen et al., 2017). Together, these data suggest that sex steroids play a neuromodulatory role in vision across taxa.

The gonadotropin system is also well documented for its ability to modulate sensory function, although most of this research focuses on central processing. GnRH3 fibers from neurons in the terminal nerve ganglia project to the inner nuclear layer, outer nuclear layer, and ganglion cells of the retina (Pfister et al., 2016; Stell et al., 1984). In most animals, gonadotropin releasing hormone (GnRH) released from the hypothalamus travels to the pituitary to stimulate release of gonadotropins (luteinizing hormone, LH, and follicle stimulating hormone, FSH). An LH surge triggers ovulation in many species and is accompanied by a suite of endocrine and physiological changes. In fishes, ovulation occurs when ova detach from the ovary lining and descend towards the urogenital opening and is associated with increased steroid production (King et al., 1994; Scott and Baynes, 1982). Prostaglandins, such as PGF2α, are also produced in the ovary in response to ovulation (Sorensen et al., 2018) and are necessary to induce egg release and spawning in fishes (Juntti et al., 2016). While these hormones are well-documented for their function in the hypothalamus-pituitary-gonad (HPG) system, they can also have neuromodulatory effects on sensory system function. GnRH and its receptors are expressed in the retina of fishes (Grens et al., 2005; Pfister et al., 2016; Stell et al., 1984), and aspects of the gonadotropin systems (e.g. LH, GnRH) can modulate activity within the retina (Stell et al., 1987; Umino and Dowling, 1991). As such, both sex steroid receptors and gonadotropin system receptors can serve as neural substrates for hormone action within the retina to promote reproductive state-dependent visual plasticity.

The social African cichlid fish *Astatotilapia burtoni* is a model system for neuroethology and sensory biology (Maruska and Fernald, 2018). Males exist as two phenotypes, dominant and subordinate, which they can rapidly and reversibly switch between. Dominant males hold territories which they vigorously defend from other males using primarily visual displays. These signals range from displays of size (flaring fins and distending jaw) to changes in coloration (black eye bar, bright body coloration) (Fernald, 1977; Fernald and Hirata, 1977). When near a ready to spawn female, males increase their courtship displays (Maruska and Fernald, 2012). Dominant males intensify their coloration during courtship, which includes a bright red humeral patch on the side of their body. They also produce a body quiver and tail waggle, so that females receive multicomponent (behavior and coloration) visual signals from males. Females need to spawn within hours of ovulation and are mouthbrooders, which after spawning will carry developing young in their mouths for ~2 wks. This quick timing and subsequent high investment in maternal care makes mate selection crucial for offspring fitness.

Here, we tested the hypothesis that reproductively-mediated plasticity exists in the eye of a social fish that uses elaborate multicomponent visual displays. Specifically, we tested the following predictions: (1) Dominant males will court (i.e. produce more visual signals towards) ovulated females more than gravid females; (2) Ovulated females will be more responsive to male courtship attempts than gravid females; (3) Animals ready to reproduce (i.e. dominant males and ovulated females) will have better visual sensitivity; and (4) neuromodulatory receptor expression in the eye is plastic and dependent on an animal's reproductive state. As predicted, males performed more courtship behaviors at ovulated females, and ovulated females were more responsive to male courtship attempts compared to gravid females. Ovulation was also associated with increased neural activation in two layers of the retina when females were visually-exposed to courting males in a behavioral context. We used electroretinograms to reveal that visual sensitivity changes with female reproductive state. Finally, we found that expression levels of reproductively important modulatory receptors in the eye change with female, but not male, reproductive state, and that their expression correlates with the increase in mate choice-like behaviors observed after ovulation. This study provides crucial evidence linking ovulation, sensory modulation, and mate choice behaviors. Together, these data indicate that females have better visual sensory perception at ovulation, a time when it is most crucial for them to select an appropriate mate.

2. Materials and methods

2.1. Experimental animals

Adult *Astatotilapia burtoni* (standard length: 41.54 ± 6.63 mm; body mass: 2.23 ± 1.02 g) were bred from a wild-caught stock from Lake Tanganyika, Africa and maintained in an environment that mimicked their natural habitat (pH = 7.6–8.0; 28–30 °C; 12 L:12D diurnal cycle). Fish were fed cichlid flakes (AquaDine, Healdsburg, CA) daily and supplemented with brine shrimp twice weekly. All experiments were performed in accordance with the recommendations and guidelines provided by the National Institutes of Health Guide for the Care and Use of Laboratory Animals, 2011. Animal care and all experimental procedures followed approved Louisiana State University or University of Minnesota Duluth IACUC protocols.

Stable dominant and subordinate males were established by placing two size-matched territorial males into a tank containing a single spawning territory with three females. In this situation, one male becomes dominant over the other within an hour and resulted in stable social states that persisted for > 30 days. Female *A. burtoni* breed year-round with a 25–30-day cycle that can be divided into three distinct phases: 1) gravid, reproductive females identified by visibly distended abdomens due to large ova, 2) mouthbrooding females, during which time they provide sole parental care to the developing embryos by brooding them in their mouths for ~12–14 days, and 3) recovering females, during which time vitellogenesis occurs and yolk deposits are replenished to prepare for the next spawning cycle. We collected mouthbrooding females 6–8 days after the onset of brooding, and gravid females based on the visibly swollen abdomens and presence of actively courting males. Gravid females were further split into ovulated or non-ovulated based on the location of the ova during dissections (released or not released from ovarian follicles, respectively). Ovulated females could also be distinguished from non-ovulated gravid females by their slightly distended jaw (in preparation for mouthbrooding) and protruding urogenital papilla.

Gonadosomatic index (GSI) was calculated for all animals during dissection, and GSI limits were set to ensure animals were within the assigned reproductive states. GSI [(gonad mass/body mass)*100] limits were as follows; gravid female: > 7.0; brooding female: < 1.0; recovering female: 2.0–7.0; dominant male: > 0.70; subordinate male: < 0.50.

2.2. Behavior trials and analysis

To examine how reproductive behaviors changed with female reproductive status, we compared interactions between dominant males and either nonovulated-gravid or ovulated females. All behavior trials occurred shortly after lights on (8:00–8:30 am). A single dominant male was placed into a 38-l tank and allowed to acclimate for 24 h. Males were behaviorally dominant for > 48 h, but GSIs ranged from subordinate to dominant values. On the morning of collection, non-ovulated-gravid females were selected from community tanks based on the presence of a distended abdomen but absence of an expanded jaw,

protruding urogenital papilla, and intensely courting males. Females were quickly injected intraperitoneally with 3 μg/g BM of prostaglandinF2α (PGF2α; 3-6 μl injected per fish) or equivalent DMSO vehicle-control, placed into the tank with the male, and allowed to interact for 30 min. Stock PGF2α (Cayman Chemical) was prepared by dissolving PGF2α powder into DMSO to a concentration of 10 μg/μl. A fresh working solution was prepared to 1 μg/μl in 0.9% saline (NaCl) on the morning of use. Interactions were recorded and later quantified for stereotypical reproductive behaviors by an observer blind to treatment. Body courtship quivers, nips, tail waggles, and leads were quantified as overt male reproductive behaviors. Bites and chases were quantified as "other" behaviors. For females, we quantified a response (positive, negative, neutral) to each male's courtship attempt. A positive response was recorded if the female oriented towards the male or followed him within 2 s of the courtship behavior. Orienting away from the male or swimming away were counted as negative responses. All other responses (i.e. remaining stationary) constituted a neutral response. At the end of the trial, both fish were sacrificed and tissue was collected and processed as described below (Section 2.5). GSI did not differ between PGF2α-injected females (8.003 ± 1.175) and vehicle-injected females (8.009 ± 0.606), or between dominant males exposed to ovulated (0.643 ± 0.210) or nonovulated (0.695 ± 0.291) females.

Male courtship behaviors vary widely and are largely dependent on female reproductive state, with males courting more intensely at females closer to spawning. Here, we used PGF2α injections to induce ovulation in females and standardize male responses. Circulating PGF2α levels spike at ovulation or shortly afterwards. While the exact mechanism is not known, we hypothesize that injections work through positive feedback via receptors at the ovaries to induce ovulation. PGF2α receptors (*ptgfr*) are also located in the brain (Juntti et al., 2016), suggesting injections may also function centrally to modify behaviors. Previously, the *ptgfr* receptor was shown to be necessary and sufficient for late-stage reproductive behaviors (i.e. circling, egg laying) in females (Juntti et al., 2016). As such, PGF2α is commonly used as an experimental method to induce ovulation and courtship interactions in fishes.

2.3. Retina neural activation via pS6 immunohistochemistry

To examine if ovulation resulted in increased neural activation in the retina, we collected eyes from ovulated females and non-ovulated gravid females. Dominant males were selected based on their bright coloration and performance of aggressive and territorial behaviors in community tanks. They were placed in a 38-l aquarium with a single spawning territory and allowed to acclimate for 24 h. On the morning of the trial, females were selected using the above criteria for ovulated females, placed immediately into the tank with the male, and allowed to interact for 45 min. Females were then quickly euthanized by rapid cervical transection, and their heads with the brains exposed and eyes loosened from surrounding tissue (to allow fixative access surrounding the eye tissue) were fixed in 4% paraformaldehyde (PFA) in 1× phosphate-buffered saline (1xPBS) overnight, rinsed in 1xPBS for 24 h, and cryoprotected in 30% sucrose for > 12 h. Immediately prior to sectioning, an eye was removed from the head, the lens removed, and the eye mounted in OCT media before sectioning at 20 μm. Sections were collected onto three alternate sets of superfrost plus microslides (VWR), air dried for 48 h, and stored at 4 °C in RNase-free conditions until staining.

We analyzed neural activation in the retina using the phosphorylated ribosome marker, pS6. Like immediate early genes, pS6 staining serves as a proxy for neural activation. Upon activation, internal signaling cascades result in phosphorylation of the S6 ribosomal protein, which leads to increased translation (Fenton and Gout, 2011; Magnuson et al., 2012; Ruvinsky and Meyuhas, 2006). Slides of sectioned eyes were stained as previously described (Butler et al., 2018) using 1:1500 dilution of phospho-S6 ribosomal protein (Ser235/236) antibody (Cell Signaling 2211S). Staining was visualized on a Nikon Eclipse Ni microscope. To consistently measure the same portion of the retina, we identified the "middle" of the eye as the point where the optic nerve leaves the eye (i.e. optic disc). We quantified the number of pS6-stained cells in both the inner nuclear layer (INL) (contains amacrine, horizontal, and bipolar cells, receives neuromodulatory inputs from the brain, and expresses modulatory receptors) and the ganglion cell layer (GCL) (axons of which form the optic nerve and relay visual information to the brain). A 600 μm curved line was drawn from the optic disc dorsally and the number of pS6 stained cells was quantified for each retinal layer of this 600 μm region. Cell number was calculated as the average number of stained cells in the quantified area across four consecutive sections.

2.4. Electroretinograms

To test for differences in visual sensitivity independent of social stimuli, we used electroretinograms (ERGs) in males, gravid females, and recovering females. Fish were initially shipped from Baton Rouge, LA to Duluth, MN in aerated bags overnight. All animals were allowed to acclimate and recover in MN for > 1 week prior to ERGs. Fish were collected from the aquatic facility and once in the recording room, only exposed to dim red light. They were then anesthetized in ice water, injected with < 0.06 cc of 0.1 mg/ml pancuronium bromide prepared in 0.9% NaCl, and placed in the recording chamber with the left eye and temporal/limbus region above the water. A tube inserted into the mouth was fed by a gravity flow water system to provide constant water circulation over the gills during the recording. A small incision (< 1 mm) was made on the limbus portion of the eye, and a silver silver-chloride recording electrode (0.2 mm diameter) was inserted into the vitreous humor behind the lens and adjacent to the retina. All electrodes were placed in approximately the same location across individuals. A reference electrode was placed subdermally between the eyes. The ERG signal was amplified (1000×, 1 Hz low pass, 3 kHz high pass, World Precision Instrument, Inc.; model DAM50; Sarasota, FL), digitized with PowerLab 4SP (AD Instruments, Castle Hill, Australia), and processed with Lab Chart7 (AD Instruments, Castle Hill, Australia) software. The recording chamber, light stimulus, and amplifier were placed in a black, opaque Faraday cage (77 × 67 × 96 cm) to eliminate outside light and electrical interference.

Fish were dark-adapted until no a-wave of the light-evoked response was present (0.5–1 h). A 100 W quartz tungsten-halogen lamp (Newport 6333, Stratford, CT) with a constant current power supply (Newport #68938) was used as a light source. An Oriel Electronic Shutter (#76994) and controller (#76995) regulated the light stimulus: square wave light pulse with a 3.0 ms delay, 3.0 ms rise time, and 5.0 ms fall time. The stimulus then passed through a monochromator (Newport #77250) and neutral density filter before a fiber optic light pipe (Newport #77632) transmitted the light to the eye. The light pipe extended to approximately 1 cm from the eye such that the entire eye was illuminated during stimulus. The amplitude of the b-wave was used to determine ERG sensitivity. The criterion response was determined initially by measuring b-wave amplitude during a 650 nm stimulus. The amount of irradiance necessary to reach ± 10% of the criterion amplitude was determined for 450-650 nm in 50 nm increments in a randomized order. For each wavelength, the neutral density filter was adjusted until the light-evoked response fell within the sensitivity criterion. Because 650 nm was used to set sensitivity criteria, responses at this wavelength could not be compared among pre-injected groups. Immediately after all wavelengths were tested, fish were quickly given an i.p. injection of PGF2α (as described above). After 30 min (in dark), we again tested the same wavelengths using the same criteria for the pre-injection baseline recordings. If during injection, the recording electrode was moved or displaced, these fish were not used. To verify that the changes in visual sensitivity observed after PGF2α injections were not an artifact of the experimental set up (e.g. longer time dark-

adapting), several fish ($N = 3$) were injected with DMSO vehicle solution instead of PGF2α and tested as described above.

It is important to note that our retinal sensitivity values do not represent absolute threshold at each wavelength tested. Rather, we used a set response (650 nm with no filter) as a "criterion response". *A. burtoni* are not very sensitive to this red colour and the response was minimal but repeatable. Based on the average response to this stimulus, we set "response criterion" as an evoked response occurring within ± 10% of the criterion response. We used this method over true threshold because system noise could potentially mask small responses. In addition, evoked responses could vary based on electrode position. Although we minimized electrode position variability across animals, using an internal response criterion as done here helped account for any variation due to electrode position. Because of this, and the fact that we use paired measurements for before and after PGF2α injection, we can be confident that the observed changes are due to differences in animal reproductive state and not the experimental set-up.

2.5. mRNA tissue collection and preparation

Because our results here showed that both neural activity and visual sensitivity vary with female reproductive state, we hypothesized that expression of neuromodulatory receptors in the eye could be a mechanism regulating this change. Therefore, we predicted that the expression of neuromodulatory receptors would also vary with female but not male reproductive state. To test this, we collected eyes from mouthbrooding (i.e. nonreproductive), nonovulated-gravid, and ovulated females, as well as from dominant and subordinate males. In addition, we collected eyes from animals used in the behavior experiments in Section 2.2. This included PGF2α-injected females (i.e. ovulated) and vehicle-injected (i.e. gravid), as well as the males exposed to them. These additional females allowed us to test the prediction that PGF2α injections would not only induce ovulation, but would also increase neuromodulatory receptor expression levels in the eye similar to naturally-ovulated females. Since male goldfish need olfactory cues from post-ovulatory females to produce visually-guided behaviors (Lord et al., 2009), we predicted that *A. burtoni* males exposed to ovulated females may have an increase in neuromodulatory receptor expression compared to males exposed to nonovulated females.

All animals used in the study were collected at the same time of day (8:00–10 AM) to minimize diurnal changes in gene expression. Animals were quickly netted from their aquaria and measured for standard length and body mass. Blood was collected from the caudal vein with heparinized 100 µl capillary tubes prior to sacrifice by rapid cervical transection. Blood was centrifuged at 8000 rpm for 10 min, and plasma was collected and stored at −80 °C until analysis.

For mRNA analysis, both eyes were quickly removed from the head by clipping the optic nerve as close to the eye as possible within ~1 min of being euthanized. The lens and any excess tissue surrounding the eye was removed, and eyes were then immediately frozen and stored at −80 °C until analysis. Gonads were removed and weighed to calculate gonadosomatic index [GSI; (gonad mass/body mass) x 100] to ensure each animal fit the group criteria. Tissue was homogenized and RNA extracted following the manufacturer's protocol (RNeasy Plus Mini Kit, Qiagen). RNA yields were calculated using spectrophotometric values to ensure consistent RNA inputs to cDNA synthesis reactions (iScript, BioRad).

2.6. Quantitative reverse transcription PCR (qRT-PCR)

Quantitative RT-PCR was used to measure mRNA expression of candidate genes from eyes. We measured expression levels of sex steroid receptors (Androgen receptors: *arα*, *arβ*; Estrogen receptors: *erα*, *erβa*, *erβb*; G protein-coupled estrogen receptor: *gper*; Progesterone receptor: *pgr*), aromatase (*aromb*), prostaglandin F2α receptor (*ptgfr*), GnRH receptors (*gnrhr1*, *gnrhr2*), LH receptor (*lhr*), and FSH receptor (*fshr*) genes. SsoFast SybrGreen supermix (BioRad) or PerfeCTa SYBR Green Fastmix (Quantabio) was used for qRT-PCR reactions with gene specific primers (Table S1). Samples from steady-state animals were run using BioRad supermix while samples from behavior trials were run with Quantabio supermix. As such, values from these two groups cannot be compared. Primers for *aromb*, *pgr*, *gper*, *ptgfr*, and the reference gene *eef1a* were designed based on sequences available in genbank (*aromb*: FJ605734; *pgr*: NM_001286327; *gper*: XM_005939507.2; *ptgfr*: NM_001286322.1; *eef1a*: XM_005919290.2). All other qRT-PCR primers were used previously (Au et al., 2006; Burmeister et al., 2007; Maruska and Fernald, 2010, 2011). Each primer pair had a single melt curve peak and amplified in a positive control (brain cDNA), and no primer set showed any amplification in the no-RT control.

qRT-PCR was performed on a CFX connect Real-Time system (BioRad) with duplicate reaction volumes of 20 µl. Reaction parameters were 95 °C for 30s, 45 cycles of 95 °C for 1 s and 60 °C for 15 s, followed by a melt curve analysis. Fluorescence thresholds for each sample were automatically measured (CFX Manager, BioRad) and PCR Miner (Zhao and Fernald, 2005) was used to calculate reaction efficiencies and cycle thresholds. The relative amount of mRNA was normalized to the geometric mean of the two reference genes (*eef1a* and *gapdh*), which were verified to not differ among female groups or between males ($P > 0.50$ for both). *Gapdh*, (glyceraldehyde 3-phosphate dehydrogenase) is a common reference gene in *A. burtoni* studies because it is ubiquitously expressed at similar levels independent of reproductive status. *Eef1a*, eukaryotic elongation factor 1-alpha, is also expressed in all cells. Other previously used reference genes for *A. burtoni* (*18s*, *rpl32*) were also tested but exhibited expression that differed across female reproductive states, making them unsuitable as reference genes for this study.

Only samples with CT values under 36 were used (average CT values = reference genes: 24–26; sex steroid receptors: 31–35; *gnrhr*s: 27–28; *lhr*: 34–35; *fshr*: 35–36). If a sample had late amplification, PCR miner was unable to accurately fit the model (i.e. "exponential phase fit error"), and these samples were removed from the dataset prior to analysis.

2.7. Hormone assays

To test for differences in circulating levels of 11-ketotestosterone, estradiol, and progestins, we performed enzyme-linked immunosorbent assays on serum collected from ovulated, nonovulated-gravid, and mouthbrooding females, as well as females injected with either PGF2α or vehicle using commercially available kits (Cayman Chemical; estradiol: 582251; 11-ketotestosterone: 582751; progestins: 582601). Serum samples were extracted three times with 200 µl ethyl ether, evaporated at room temperature in a fume hood, and reconstituted in assay buffer (1:35 dilution). Kit protocols were strictly followed, and each plate was read in triplicate at 405 nm. Concentrations were determined based on standard curves. Each sample was assayed in duplicate for each hormone, and all samples fit on a single plate for each hormone. Kits have been previously validated for this species (Maruska and Fernald, 2010). Intra-assay CVs were 9.94%, 9.27%, and 10.10% for 11-KT, E2, and P4, respectively.

2.8. Statistics

To test for differences in visual sensitivity between different reproductive states, we used a 2-way repeated measures ANOVA with wavelength as the repeated variable. To test for differences in mRNA levels of neuromodulatory receptors between reproductive states, we used analysis of covariance (ANCOVA) with body size (SL and BM) as covariates. All pairwise comparisons were done using Tukey's tests. Correlations were assessed using Pearson product moment tests when the data was normally distributed or Spearman rank tests when normality was not met. Iglewicz and Hoaglin's robust test for multiple

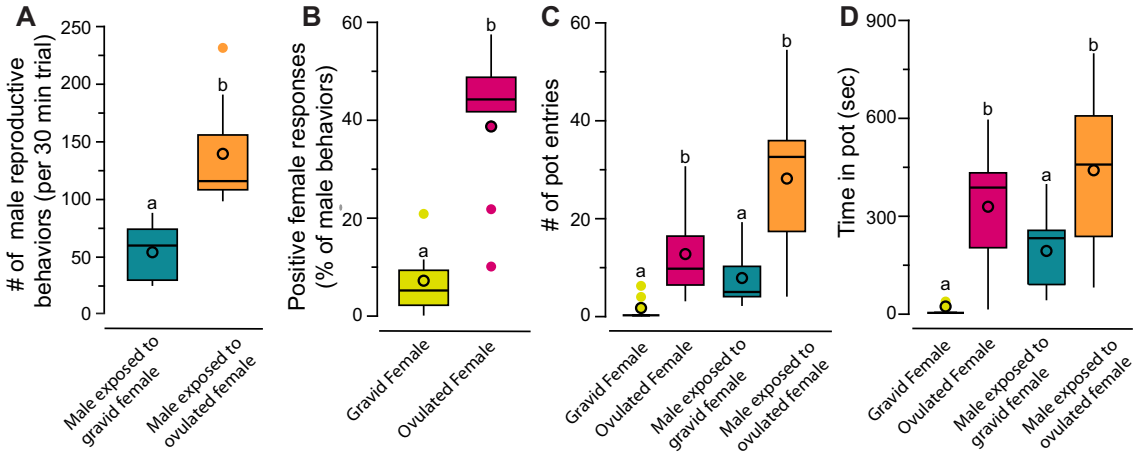

Fig. 1. Male and female reproductive behaviors vary with female ovulation status. (**A**) Males exposed to ovulated females (orange) perform more courtship behaviors than males exposed to gravid females (blue). (**B**) Ovulated females (pink; PGF2α-injected, see methods for details) respond to male courtship behaviors more than gravid females (yellow), indicative of greater affiliation. (**C-D**) Ovulated females and males exposed to them enter the spawning territory more and spend more time in the pot than gravid females and males exposed to them. N = 7 for all groups. Tukey's boxplots are used for data representation. Data median is represented by a line and data mean by an open circle, the box extends to the furthest data points within the 25th and 75th percentile, and whiskers extend to the furthest data points not considered outliers. Absence of whiskers indicates absence of data points outside of the 25th/75th percentile. Outliers (defined in Tukey's boxplots as data points outside 1.5× the interquartile range) are represented by closed circles and are not reflective of statistical outliers (see methods for details). Different letters represent statistical significance at $P < 0.05$. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

outliers was used to identify statistical outliers (Iglewicz and Hoaglin, 1993). Statistical outliers were removed from datasets prior to analysis. "Outliers" depicted as filled circles in figures (see Fig. 1 for details) fall outside of 1.5× the interquartile range (as per Tukey's box plots), but do not reflect statistical outliers as tested above. While Bonferroni reduces the chance of type I errors, it also reduces statistical power and increases the chance of type II errors, potentially masking biologically relevant effects. Instead, corrections for multiple comparisons were done by calculating a false discovery rate via Bejamini-Hochberg procedure with a strict FDR of 0.05. While we present exact *p*-values in text and tables, only two values were no longer significant after FDR corrections. These p-values are indicated in tables and the text. Effect size was calculated for all tests and presented throughout the text and tables. For ANOVAs, eta values were calculated as the sum of squares for the effect divided by the total sum of squares. This produced values between 0 and 1, with larger values reflecting a higher effect size. For comparisons between two groups and post-hoc testing, we calculated effect size as Hedge's g (as done in Ellis, 2010). Hedge's g, as opposed to Cohen's d, was used due to unequal sample sizes among the groups. All plots are represented as mean ± SEM or Tukey's box-plots. All statistics were performed in SPSS or SigmaPlot.

3. Results

3.1. Ovulated females perform more affiliative behaviors

Males exposed to ovulated females performed more overt reproductive behaviors (i.e. body quiver, tail waggle, lead to pot) than those in the presence of a gravid female ($P = 0.009$; g = 1.544; Fig. 1A). These male behaviors all include visual stimuli that females are likely to detect and use for mate choice. In addition, ovulated females positively respond to male courtship behaviors more than gravid females ($P < 0.001$; g = 2.259; Fig. 1B). Both ovulated females, and males exposed to them, enter the spawning territory more and spend more time in the territory than gravid females and males exposed to gravid females (female pot time: $P < 0.001$, g = 2.181; female pot entries: $P = 0.012$, g = 0.483; male pot time: $P = 0.005$, g = 1.729; male pot entries: $P = 0.026$, g = 1.275; Fig. 1C-D). Together, these data indicate that visual signaling is used during reproductive interactions, and that production of visual stimuli from males and the female's behavioral response to it varies with her ovulation status.

3.2. Retina neural activation is higher in ovulated females

Given the behavioral importance of male-female visual signaling

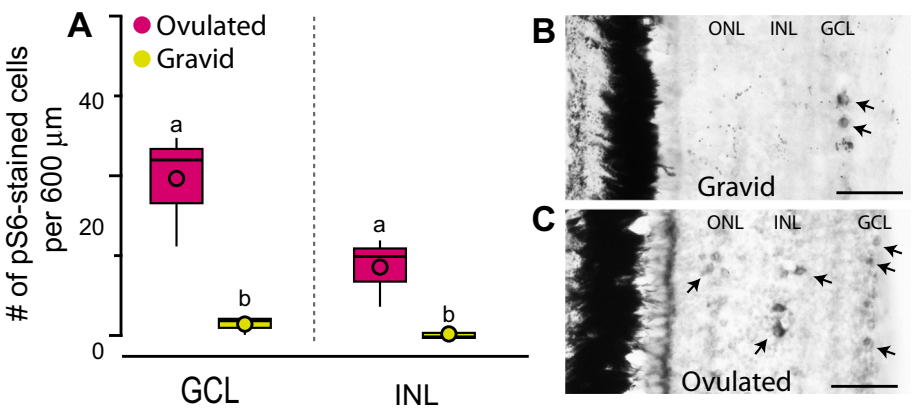

Fig. 2. Neural activation in the retina of females visually exposed to courting males varies with ovulation status. (**A**) Ovulated females (red) have more pS6 staining in the ganglion cell layer (GCL) and inner nuclear layer (INL) of the retina compared to gravid (yellow) females. Representative photomicrographs of pS6 staining in retina of gravid (**B**) and ovulated (**C**) females exposed to courting males. Ovulated females also have faintly-stained cells in the outer nuclear layer (ONL). Arrows in B–C point to pS6-stained cells, but not all cells are indicated. Scale bars in B–C represent 25 µm. $N = 4$ for all groups. See Fig. 1 for box plot descriptions. Different letters represent statistical significance at $P < 0.05$. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

related to ovulation status, we hypothesized that ovulation may also result in greater activation in the retina when females are exposed to visual courtship signals from males (Fig. 2, S2). To test this, ovulated (natural and PGF2α-injected) and gravid (natural and vehicle-injected) females were exposed to courting males, and their eyes were collected and stained for the neural activation marker, pS6. We observed pS6 stained cells in the inner nuclear layer (INL) and ganglion cell layer (GCL) of the retina in all fish. Ovulated females (natural and PGF2α-injected) have greater activation than non-ovulated females in both INL and GCL (ANOVA; GCL: $F_{3,12} = 26.885$, $P < 0.001$, eta = 0.900; INL: $F_{3,12} = 17.575$, $P < 0.001$, eta = 0.854; post-hoc $P < 0.001$ for all ovulated vs non-ovulated comparisons). Within ovulated females, PGF2α injected females had greater activation in the GCL and INL than naturally ovulated females (GCL: $P < 0.001$, g = 2.378; INL: $P = 0.003$, g = 0.973), possibly due to the high concentration used for PGF2α injections. This greater activation in ovulated females may result from increased visual stimuli from dominant males that intensify their courting behaviors towards ovulated females compared to gravid females. The higher neural activation in ovulated females when they are exposed to dominant males suggests functional differences related to visual detection or discrimination.

3.3. Visual sensitivity varies with female reproductive state

To further examine how female visual sensitivity changes with reproductive state, independent of the presence of a male stimulus, we used electroretinograms to measure sensitivity at several wavelengths within their spectral range (450 nm, 500 nm, 550 nm, 600 nm, and 650 nm; Fig. 3). The irradiance needed to evoke a criterion response at each wavelength was used as a measure of spectral sensitivity for the purpose of this study. Peak sensitivity was observed at 550 nm for all animals (Fig. 3C); however, sensitivity varied with reproductive state in a wavelength-dependent manner (2 way RM ANOVA; status: $F_{1,17} = 6.048$, $P = 0.028$, eta = 0.061; wavelength: $F_{4,79} = 56.733$, $P < 0.001$, eta = 0.569; status X wavelength: $F_{4,79} = 4.075$, $P = 0.006$, eta = 0.041). Overall, gravid females had better sensitivity compared to recovering females, but post-hoc tests reveal that this difference in sensitivity was only at 500 nm ($P = 0.006$, g = 4.081) and 550 nm ($P < 0.001$, g = 5.357).

The effect of ovulation on spectral sensitivity was determined by injecting recovering females, gravid females, and males with PGF2α after an initial baseline ERG recording and retesting of all fish 30-min after injection (Fig. 3A–B). Ovulation status was verified by dissection in females after the post-injection recordings. Compared with pre-injection recordings, PGF2α-injected gravid females (i.e. ovulated) had significantly better visual sensitivity ($F_{1,79} = 10.756$, $P = 0.013$, eta = 0.062; Fig. 3E). This increased sensitivity was not observed in PGF2α-injected recovering females or males (Recovering: $F_{1,49} = 0.124$, $P = 0.742$, eta < 0.01; Males: $F_{2,29} = 0.496$, $P = 0.554$, eta < 0.01; Fig. 3D, S1B) or in gravid females injected with DMSO vehicle ($F_{2,29} = 0.893$, $P = 0.444$, eta < 0.01; Fig. 3F). To further analyze changes in sensitivity, we calculated the percent change between pre- and post- PGF2α injection recordings (Status: $F_{2,79} = 3.843$, $P = 0.049$, eta = 0.340, Fig. 3G). Gravid-PGF2α injected females had a significantly greater increase in sensitivity compared to PGF2α-injected recovering females and vehicle-injected females independent of wavelength ($P = 0.671$, g < 0.001). Interestingly, the percent change in spectral sensitivity correlated with female GSI (R = -0.760; $P = 0.007$; Fig. 3H) such that the higher the GSI, the better the visual sensitivity (i.e. greater the negative percentage change).

3.4. Neuromodulatory receptor expression varies with female but not male reproductive state

To test for differences in expression levels of reproductively important modulatory receptors that may contribute to the observed changes in visual sensitivity, we used qPCR to measure the following transcripts in the eyes of males and females of various reproductive and social states (ANCOVAs; Table 1, Fig. 4A): estrogen receptors (*erα*, *erβa*, *erβb*, *gper*), androgen receptors (*arα*, *arβ*), progesterone receptor (*pgr*), prostaglandin F receptor (*ptgfr*), gonadotropin system receptors (*gnrhr1*, *gnrhr2*, *lhr*, and *fshr*) and aromatase. *Arα*, *arβ*, *erα*, *erβa,* and *erβb* expression was higher in the eyes of ovulated females than those from nonovulated-gravid and brooding females (*arα*: $P < 0.001$; *arβ*: $P < 0.001$; *erα*: $P < 0.001$; *erβa*: $P < 0.001$; *erβb*: $P < 0.001$; see Table 1 for post-hoc statistics and effect size). *Gper* expression levels differed among all three female groups ($P < 0.001$). Ovulated females had higher *gper* expression than nonovulated-gravid and brooding females while nonovulated-gravid females had higher expression than brooding females. Progesterone receptor expression was almost four-fold higher in ovulated females compared to nonovulated-gravid and brooding females ($P < 0.001$). *Aromb* expression differed among all three groups of females ($P < 0.001$). Ovulated females had higher expression than nonovulated-gravid females and brooding females, and nonovulated-gravid females had higher expression than brooding females. All females had similar levels of *ptgfr* expression in the eye ($P = 0.125$). Ovulated females had higher expression of *gnrhr1*, *lhr,* and *fshr* compared to nonovulated-gravid and mouthbrooding females (*gnrhr1:* $P = 0.001$; *lhr*: $P < 0.001$; *fshr*: $P = 0.003$). Expression of *gnrhr2* was similar between ovulated and nonovuated gravid females, but higher than in mouthbrooding females ($P = 0.005$). In summary, ovulated females had higher expression of most reproductive neuromodulatory receptors compared to nonovulated-gravid and brooding females, indicating that ovulation may increase their expression levels in the eye.

To verify that ovulation resulted in an increase in the above-mentioned transcripts, we injected gravid females with the post-ovulatory hormone PGF2α or a DMSO vehicle control. Females injected with PGF2α had higher expression levels of some reproductively important genes in the eye compared to vehicle-injected females (see Table 2 for ANCOVA statistics and effect size calculations; Fig. 4B). Expression levels of *arα*, *arβ*, *erα*, *erβb, pgr*, *aromb*, and *lhr* were higher in PGF2α-injected females (*arα*: $P = 0.010$; *arβ*: $P = 0.015$; *erα*: $P = 0.010$; *erβb*: $P = 0.032$; *pgr*: $P = 0.005$; *aromb*: $P = 0.004$; *lhr*: $P = 0.008$), but there was no difference in *erβa*, *gper*, *ptgfr*, *gnrhr1*, *gnrhr2*, or *fshr* (*erβa*: $P = 0.474$; *gper:* $P = 0.554$; *ptgfr*: $P = 0.409$; *gnrhr1:* $P = 0.236$; *gnrhr2:* $P = 0.079$; *fshr:* $P = 0.533$).

In contrast to females, there were no differences in expression levels of any of the measured genes between dominant and subordinate males (Table 1; Fig. S3). Males exposed to PGF2α-injected females had higher expression of *erβb* ($P = 0.047$, but not significant after Benjamini-Hochberg corrections), but no other neuromodulatory receptors when compared to males exposed to vehicle-injected females (Table 2). Thus, females, but not males, show reproductive-state plasticity in expression levels of modulatory receptors in the eye. Changes in expression of neuromodulatory receptors may underlie observed changes in retina neural activity and visual sensitivity, but direct testing of this hypothesis is still needed.

3.5. Gonadosomatic index and circulating sex steroid levels

Gonadosomatic index (GSI), a measure of reproductive investment, was calculated for all animals and levels of circulating sex steroids were measured for a subset of females. GSI was ~10-fold higher in gravid than brooding females ($P < 0.001$, g = 10.600), but GSI did not differ between ovulated and nonovulated-gravid females ($P = 0.563$, g = 1.428). GSI was almost two-fold higher in dominant males than in subordinate males ($P < 0.001$, g = 4.232). Ovulated females had higher levels of 11-KT and estradiol compared to both nonovulated-gravid and mouthbrooding females (ANCOVA; 11-KT: $P = 0.006$, eta = 0.482; E_2: $P = 0.001$, eta = 0.472; Fig. S4), but there were no differences in circulating levels of progestins ($P = 0.052$, eta = 0.292).

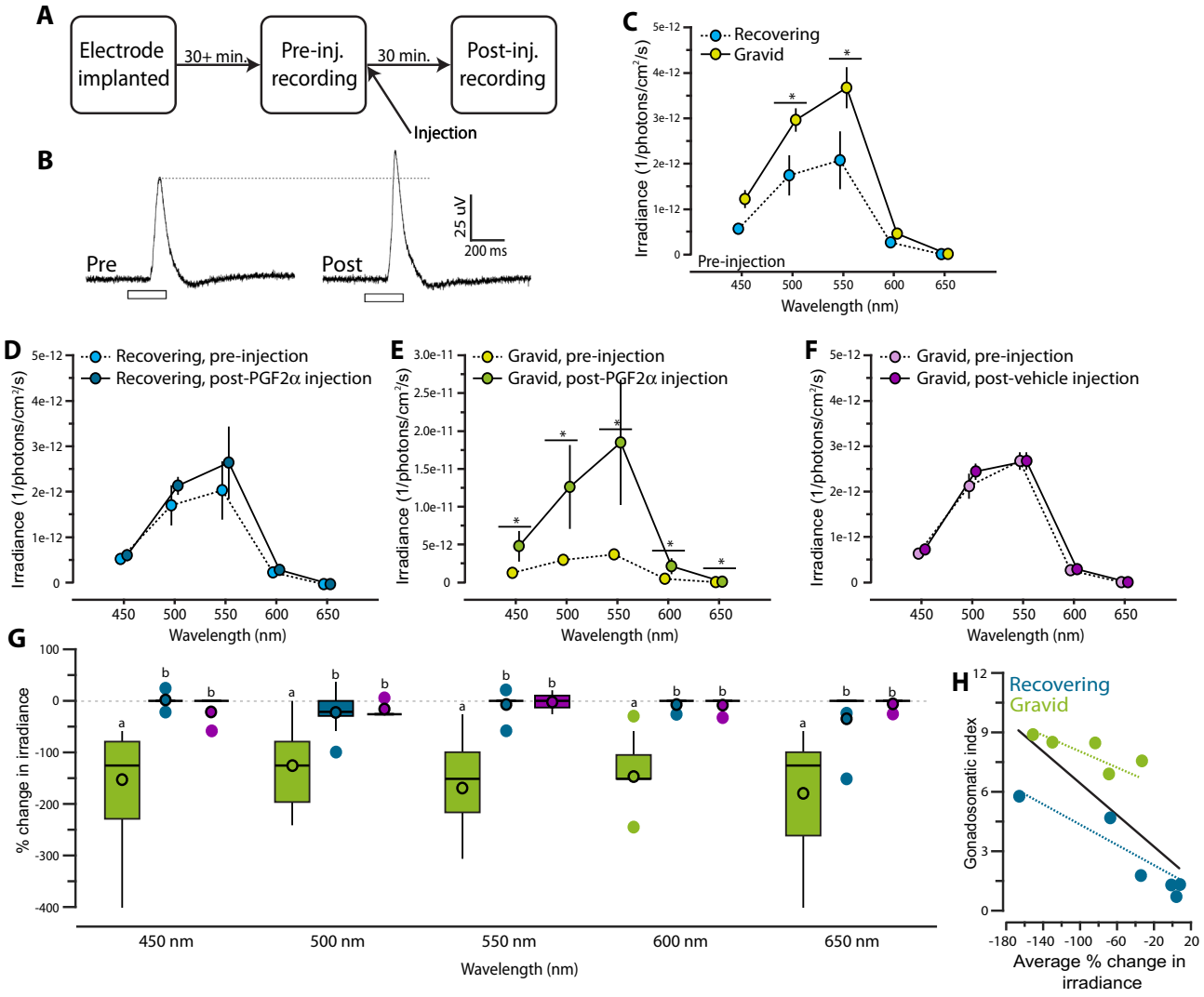

Fig. 3. Visual sensitivity measured by electroretinograms changes with female reproductive state. (**A**) Schematic of timeline for dark-adapting, baseline recordings, injections, and post-injection ERG recordings. (**B**) Representative waveform of ERG trace from a gravid female during baseline and post-PGF2α-injection conditions. White bar indicates presentation of stimulus: 550 nm with a 0.5 neutral density filter applied. (**C**) Gravid females (N = 8) have greater spectral sensitivity at 500 and 550 nm compared to recovering, non-reproductive females (*N* = 7). (**D–E**) Spectral sensitivity does not change after recovering females are injected with PGF2α (N = 6), but gravid females (*N* = 5) have increased sensitivity at several wavelengths associated with male courtship coloration and displays. (**F**) Injecting gravid females with a vehicle control (DMSO; N = 3) had no impact on spectral sensitivity. (**G**) Changes in sensitivity were calculated as a percentage change from initial baseline values. Gravid females injected with PGF2α had a significantly greater change in sensitivity compared to recovering females injected with PGF2α and vehicle control gravid females. (**H**) The average percent change in sensitivity (all 5 wavelengths averaged together) in PGF2α-injected females negatively correlates with gonadosomatic index both within each reproductive state (green = gravid, blue = recovering) and all females combined (black line). C—F are plotted as mean ± s.e.m. See Fig. 1 for box plot descriptions. Different letters represent statistical significance at $P < 0.05$. Asterisks in C, E represent post-hoc differences between groups within each wavelength. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

Interestingly, nonovulated-gravid and brooding females had similar levels of circulating steroids (Tukey's post hoc: 11-KT: $P = 0.509$, g = 0.566; E_2: $P = 0.232$, g = 0.987). Of the eight females injected with PGF2α, seven had partially ovulated by the end of the 30-min trial. However, none of the vehicle-injected females ovulated during the trial despite the presence of a courting male. Injecting females with PGF2α had no impact on circulating levels of 11-KT ($P = 0.356$, g = 0.621) or E_2 ($P = 0.449$, g = 0.609), but PGF2α-injected females had lower levels of circulating progestins than vehicle-injected females ($P = 0.040$, g = 0.997; Fig. S4).

3.6. Gonadotropin system receptors, but not sex steroid receptors, correlate with female affiliative behaviors

To examine whether levels of modulatory receptors in the eye were related to reproductive behaviors in individual females, we tested for correlations between mate choice-like behaviors (i.e. positive responses to male courtship, time in spawning territory) and expression levels. *Gnrhr2* and *lhr* levels positively correlate with positive female responses to male courtship behaviors (Pearson's correlation; *gnrhr2*: $R = 0.616$, $P = 0.025$; *lhr*: $R = 0.705$, $P = 0.007$) and time spent in the spawning territory (*gnrhr2*: $R = 0.716$, $P = 0.006$; *lhr*: $R = 0.685$, $P = 0.010$; Fig. 5). There were no other significant correlations between gene expression levels and behaviors (Table S2). There were also no correlations between circulating steroid levels and behaviors, but this is not surprising given that PGF2α injections do not cause an increase in estradiol or 11-KT levels in this time frame. Together, the PGF2α injection experiments confirm that females increase their affiliative behaviors towards males at ovulation and verify that ovulation status, not just gravidity, mediates expression of neuromodulatory receptors in the eye

Table 1
Planned comparisons within sex to test for reproductive-state differences in expression levels of reproductively important genes in the eye was done using ANCOVAs with body size as a co-variate. Within females, ovulated (Ov), gravid (Gr), and mouthbrooding (Br) females were compared using Tukey's post-hoc test. In males, dominant and subordinate individuals were compared. F statistic and *P* values for each comparison are shown on the top lines. Degrees of freedom are shown below F statistics and effect size is indicated below P values. Bold indicates significance at $P < 0.05$. All indicated P values remained significant after Benjamini-Hochberg corrections.

	Females					Males	
	F	P	Ov vs Gr	Ov vs Br	Gr vs Br	F	P
arα	70.738	**< 0.001**	**< 0.001**	**< 0.001**	0.454	0.677	0.418
	2, 27	0.838	4.444	4.516	0.307	1, 28	0.287
arβ	13.799	**< 0.001**	**0.002**	**< 0.001**	0.157	0.257	0.617
	2, 27	0.524	1.485	2.156	0.876	1, 28	0.460
erα	47.241	**< 0.001**	**< 0.001**	**< 0.001**	0.614	3.090	0.584
	2, 26	0.815	3.608	4.557	0.164	1, 28	0.004
erβa	35.204	**< 0.001**	**< 0.001**	**< 0.001**	0.542	0.048	0.828
	2, 26	0.768	3.216	3.896	0.268	1, 28	0.514
erβb	34.688	**< 0.001**	**< 0.001**	**< 0.001**	0.371	2.820	0.065
	2, 26	0.783	2.847	3.936	0.462	1, 25	0.280
gper	31.884	**< 0.001**	**< 0.001**	**< 0.001**	**0.013**	1.090	0.306
	2, 27	0.692	1.932	3.448	1.260	1, 29	0.431
pgr	56.045	**< 0.001**	**< 0.001**	**< 0.001**	0.268	0.302	0.584
	2, 27	0.823	3.145	4.635	0.886	1, 27	1.254
aromb	11.137	**< 0.001**	**0.025**	**< 0.001**	**0.003**	0.966	0.335
	2, 27	0.469	0.417	4.113	1.331	1, 28	0.824
ptgfr	2.275	0.125				3.370	0.079
	2, 27	0.165				1, 26	0.477
gnrhr1	11.058	**0.001**	**0.001**	**< 0.001**	0.662	0.818	0.374
	2, 24	0.518	1.857	2.312	0.253	1, 28	0.302
gnrhr2	6.740	**0.005**	0.982	**0.010**	**0.005**	0.760	0.391
	2, 26	0.372	0.039	1.635	1.542	1, 28	0.459
lhr	15.807	**< 0.001**	**< 0.001**	**< 0.001**	0.899	0.241	0.628
	2, 24	0.633	1.773	2.437	0.527	1, 28	0.457
fshr	9.029	**0.003**	**0.002**	**0.002**	0.789	0.517	0.482
	2, 17	0.618	1.850	2.208	0.979	1, 20	0.220

of females. Together, these data indicate that visual sensitivity varies with female reproductive state, likely in a hormone-dependent manner (Fig. 6).

4. Discussion

Using an integrative approach that incorporated measures at behavioral, hormonal, neurophysiological, and molecular levels, we demonstrate that the visual system of the social cichlid fish *A. burtoni* shows reproductive state-dependent plasticity in females, but not in males. Visual communication is vital for many life tasks, including courtship and reproduction. As far back as Tinbergen's initial experiments on sign stimuli with stickleback fish (Tinbergen, 1952), the importance of visual signals to courtship and spawning has been known. In the African cichlid fish *Astatotilapia burtoni*, males produce elaborate courtship displays towards females that include intensification of body and fin coloration (Fernald, 1977; Fernald and Hirata, 1977; Maruska and Fernald, 2018). These can include bright yellow or blue body coloration, red humeral patches, black eyebars, vertical banding on the trunk, and red/orange/yellow spots on the dorsal, caudal, and anal fins. Although their behaviors are multisensory in nature, their courtship displays have strong visual components. Body quivers, tail waggles, and leads may reveal aspects of male fitness to females while also enticing them into the male's spawning territory. Across taxa, males typically produce the courtship displays while females use information from these signals to choose their mate(s) (Ryan, 1990). Males also increase courtship displays in the presence of ready to reproduce females. Male *A. burtoni* will perform courtship based on the female visual cues alone, but chemosensory signals further increase male courtship behaviors (Field et al., 2018). Thus, female ovulation status is likely conveyed to the males via chemosensory channels (Fujita et al., 1991; Li et al., 2017; Sorensen et al., 2018). We also know females become more behaviorally receptive towards male courtship as their reproductive cycle progresses (Kidd et al., 2013), but know relatively little about how their ability to detect male visual courtship signals might vary with reproductive state. While previous studies found that androgens can have rapid effects on visual sensitivity in male fishes (Shao et al., 2014; Yue et al., 2018), whether the female visual system also has hormone-dependent plasticity is relatively unexplored. Thus, our research demonstrating ovulation-specific plasticity in the visual system of female cichlids advances the comparative field of hormone/reproductive-state influences on sensory function.

We observed greater neural activation in the retina of ovulated females compared to gravid females when exposed to dominant males. PS6, a marker for neural activation, was observed in the inner nuclear layer (INL) and ganglion cell layer (GCL) of the retina. Similarly, when exposed to a female stimulus, male goldfish injected with testosterone have more *cfos*-expressing cells in the INL and GCL compared to vehicle-injected males (Yue et al., 2018). The INL is comprised of amacrine, bipolar, and horizontal cells, all of which fine-tune and modify signals from photoreceptors. Importantly, axons from cell bodies in the GCL form the optic nerve and project to visual processing regions in the brain. Hormone-mediated activation in these layers indicate plasticity at the level of the eye, such that fish ready to spawn have modified signaling to facilitate detection and/or fine-scale discrimination of salient visual signals.

Reproductive state-dependent plasticity in female *A. burtoni* was observed at all wavelengths tested from 450 (blue) to 650 (orange-red) with the largest shift at the 500-550 nm range (yellow-green). Male *A. burtoni* display several visual signals during courtship, such as a bright orange-red humeral patch and increased yellow or blue body coloration. When an *A. burtoni* female ovulates, she has approximately 24 h to choose a male (or males) and spawn before she will release/deposit and pick up her eggs in her mouth, which will go unfertilized without a male. Several dominant males will court a single female, so this female must be able to adequately detect and process visual information to make a choice about which male(s) she will spawn with. Because these females invest heavily in offspring care via mouthbrooding, choice of a high-quality male is important. As such, it is crucial for ovulated females to effectively detect males' courtship displays. Modulation of sensory function with ovulation would ensure that females have enhanced detection mechanisms when needed most; at a time crucial for selecting the best mate. Appropriate mate selection is even more critical in species such as *A. burtoni* in which females invest heavily in maternal care (2-week brooding period characterized by no feeding and reduced body mass followed by 1–2 days of care for free-swimming fry).

The idea of hormone-dependent changes in visual sensitivity is not unprecedented. When tested during the summer spawning months, female stickleback fish are more sensitive to red light than are males (Cronly-Dillon and Sharma, 1968). But when tested during winter (non-breading season) females are less sensitive, such that males and females had similar sensitivity to red wavelengths. Importantly, these red wavelengths roughly correspond to the red-coloration of male stickleback bellies exhibited during courtship and spawning (Cronly-Dillon and Sharma, 1968). Electroretinograms were later used to confirm that sensitivity to 650-700 nm light (i.e. red) varies with photoperiod and is androgen dependent in sticklebacks. In addition, male stickleback that are either sexually mature or castrated and supplemented with 11KT have higher expression of the red-sensitive opsin gene (*lws*) in the eye compared to sexually immature and castrated males (Shao et al., 2014). Male goldfish injected with testosterone also have increased neural activation in the retina and a higher response to a white light stimulus (Yue et al., 2018). This response in male goldfish, however, was found to be mediated by ERβ receptors, not androgen receptors. Here, we found that reproductively-important neuromodulatory receptors in the

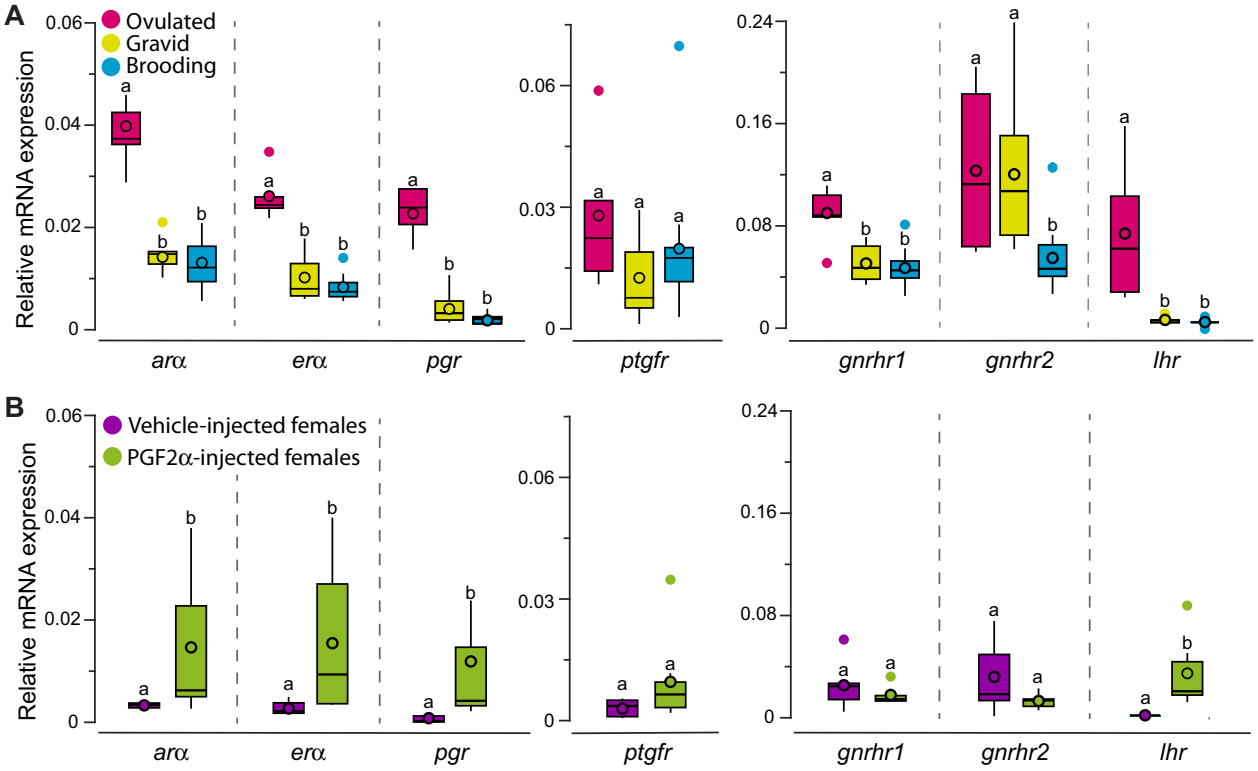

Fig. 4. Neuromodulatory receptor expression in the eye varies with female ovulation status. (**A**) Ovulated-gravid females (red; $N = 6$) have higher expression of sex steroid receptors, prostaglandin F2α receptor, and gonadotropin system receptors in the eyes than nonovulated-gravid (yellow; $N = 8$), and brooding (blue; $N = 14$) females. (**B**) PGF2α injections to mimic/induce ovulation selectively affects expression of neuromodulatory receptors in the eye, such that expression of sex steroid receptors and *lhr* are higher in PGF2α-injected females (green; N = 7) compared to vehicle injected females (purple; N = 7). Only a subset of tested genes is shown here. For full list of results, see text and Table 1. Y-axis in B is set to the same as A for ease of comparison. See Fig. 1 for box plot descriptions. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

eye vary with female reproductive state but not with male social status. Further, females had an ovulation-dependent increase in most of these receptors, indicating that this plasticity is dependent on endogenous hormonal state. Mosquitofish exposed to waterborne estrogen had altered levels of androgen receptors and the long-wavelength sensitive opsin in the eye, indicating that even exogenous hormones, in addition to endogenous ones, can alter expression of reproductive neuromodulatory receptors and other components of the visual system (Friesen et al., 2017). Our study is consistent with the hypothesis that reproductive- and hormonal-state modulation of vision at the level of the retina is conserved across vertebrates. Our data, combined with previous studies, reveals greater visual sensitivity within 30 min. of induced ovulation, which indicates that the visual system can be modulated by hormonal state, and that these hormones can have rapid effects. These rapid visual changes have important behavioral significance in any species that must choose mates quickly within a limited period of reproductive receptivity.

Sex steroids are potent modulators of sensory function across taxa. In the auditory system, for example, progestins negatively affect hearing thresholds in elderly women (Guimaraes et al., 2006), and seasonal changes in the auditory system are estrogen-dependent in the midshipman fish such that individuals are more sensitive during the breeding season (Sisneros et al., 2004). In song birds, estrogens have rapid effects on hearing and neuroestrogens can affect processing of auditory stimuli in the brain (Krentzel and Remage-Healey, 2015; Maney and Pinaud, 2011; Remage-Healey et al., 2013). In the visual system, research has primarily focused on changes in perception of visual stimuli that occurs centrally, rather than on detection in the retina. However, sex steroid receptors and gonadotropin system components have been isolated from eyes of fishes (Begay et al., 1994; Behrens et al., 1993; Grens et al., 2005; Mangiamele et al., 2017; Maruska and Tricas, 2007; Servili et al., 2012; Tchoudakova et al., 1999) and mammals (Cascio et al., 2007; Kobayashi et al., 1998; Ogueta et al., 1999; Wirsig-Wiechmann and Wiechmann, 2002), providing the neural substrate for modulation of visual processing within the eye.

It is well accepted that GnRH can modulate visual processing. GnRH3 neurons in the terminal nerve project to the retina in many fishes (Behrens et al., 1993; Maruska and Tricas, 2007; Münz et al., 1982; Oka, 1992; Stell et al., 1984). Their fibers form a dense plexus between the inner nuclear and inner plexiform layers, and nerve terminals are found throughout the inner nuclear layer, outer nuclear layer, and ganglion cell layer where they synapse with dopaminergic interplexiform cells and ganglion cells (Pfister et al., 2016; Stell et al., 1984). Application of LHRH agonists increase ganglion cell activity (Stell et al., 1987), and GnRH stimulates release of dopamine from interplexiform cells in the retina (Umino and Dowling, 1991). Visual social cues alter activity of GnRH3 neurons in the terminal nerve (Ramakrishnan and Wayne, 2009), and GnRH mediates olfactory and visual inputs via the terminal nerve to promote spawning behaviors in zebrafish (Li et al., 2017). The correlation of mate choice behaviors and expression of gonadotropin system receptors in the eye of *A. burtoni* further supports the GnRH system as a crucial component of visual communication during courtship and spawning in fishes.

Increases in neuromodulator receptor expression in the eye, affiliative behaviors, neural activation, and visual sensitivity were all linked to ovulation in female *A. burtoni*. Males showed no evidence for visual plasticity associated with their reproductive state and social status. Dominant and subordinate males had similar levels of all reproductively-related genes and did not show sensitivity changes following PGF2α injections. This further supports the idea that hormonal

Table 2
Comparison of expression levels of reproductively-important genes in the eyes was done using ANCOVAs with body size as a co-variate. Females: PGF2α-injected vs DMSO-vehicle injected. Males: males exposed to PGF2α-injected females vs males exposed to DMSO-vehicle injected females. F statistic and P values for each comparison are shown on the top lines. Degrees of freedom are shown below F statistics and effect size is indicated below P values. Bold indicates significance at $P < 0.05$. Only a subset of genes was measured in males. * indicates P-value not significant after Benjamini-Hochberg corrections (5%).

	Females		Males	
	F	P	F	P
arα	10.159	**0.010**	0.248	0.146
	1, 13	1.016	1, 13	0.882
arβ	8.652	**0.015**	0.791	0.395
	1, 13	0.925	1, 13	0.451
erα	10.086	**0.010**	0.571	0.467
	1, 13	1.035	1, 13	0.395
erβa	0.551	0.475	4.059	0.072
	1, 13	0.404	1, 13	1.069
erβb	6.230	**0.032**	5.113	**0.047***
	1, 13	0.838	1, 13	1.271
gper	0.374	0.554	0.018	0.896
	1, 12	0.481	1, 13	0.075
pgr	12.931	**0.005**	2.859	0.122
	1, 13	1.029	1, 13	0.951
aromb	13.939	**0.004**	2.450	0.149
	1, 13	1.048	1, 13	0.869
ptgfr	0.744	0.409		
	1, 13	0.578		
gnrhr1	1.615	0.236		
	1, 11	0.530		
gnrhr2	3.386	0.079		
	1, 12	0.801		
lhr	11.021	**0.008**		
	1, 13	1.127		
fshr	0.425	0.533		
	1, 8	0.256		

changes related to ovulation, which are absent in males, is mediating female visual plasticity. Ovulation is a complicated neuroendocrine process involving several hormonal pathways. The gonadotropin system is the key regulator with an LH surge from the pituitary triggering ovulation in most taxa. Downstream changes involve steroidogenesis from the ovaries, evident by increases in circulating sex steroids. In female goldfish, an increase in circulating PGF2α was observed within 15 min of ovulation, peaked approximately 9 h later, and then levels returned to baseline after fish released eggs and spawned (Sorensen et al., 2018). PGF2α is a useful tool in neuroethology research for inducing reproductive interactions in fishes. When injected either into the brain or body cavity, PGF2α induces female affiliative behaviors similar to those seen in gravid, ready-to-spawn females (Juntti et al., 2016). PGF2α is thought to bind receptors in the preoptic area of the brain (and potentially other regions) to induce reproductive behaviors. While levels of the PGF2α receptor in the preoptic area of the brain increase as *A. burtoni* females get ready to spawn (Juntti et al., 2016), *ptgfr* levels in the eye were similar among ovulated, gravid, and brooding females. In fact, *ptgfr* was the only measured gene in our study that did not differ among female reproductive states. However, the fact that receptor levels were similar in all females indicates that the observed post-PGF2α injection change in gravid females was not due to a direct effect of PGF2α in the eye. More likely, PGF2α injections caused indirect effects mediating visual sensitivity (e.g. via actions in the eye, brain, pituitary, or at the ovaries). In addition, the PGF2α injections did not affect circulating levels of 11-KT or estradiol, indicating that the increased visual sensitivity was also not from changes in circulating steroid levels. We propose that while PGF2α likely acts at the level of the ovaries to stimulate ovulation, it could also act via the preoptic area in the brain with potential effects on the gonadotropin system. This is also supported by the positive correlation between female affiliative behaviors and gonadotropin system receptor, but not steroid receptor, expression in the eye.

The influence of reproductive state on vision is not limited to fishes. The link between hormones and ocular function and health is well demonstrated (Affinito et al., 2003; Vajaranant et al., 2010). Studies in humans and other mammals have demonstrated reproductive-state changes in visual processing and preference (Little, 2013). By showing women on different days of their menstrual cycle images of men, one study found that women have an increased preference for “masculine” features around the time of ovulation (Little, 2013; Little et al., 2007; Penton-Voak et al., 1999; Johnston et al., 2001). Visual plasticity in the eye is likely a widespread phenomenon, but limited research has tested for hormone-mediated visual plasticity. This study provides crucial evidence linking ovulation, sensory plasticity, and mate choice. In seasonally breeding animals, whose endocrine profiles change across the year, hormone-dependent visual plasticity allows animals to enhance sensory capabilities when behavioral decisions are crucial for successful reproduction. The ability to discriminate and choose the correct or best mate specifically at ovulation has important consequences for mate choice and is adaptive for reproductive fitness across taxa.

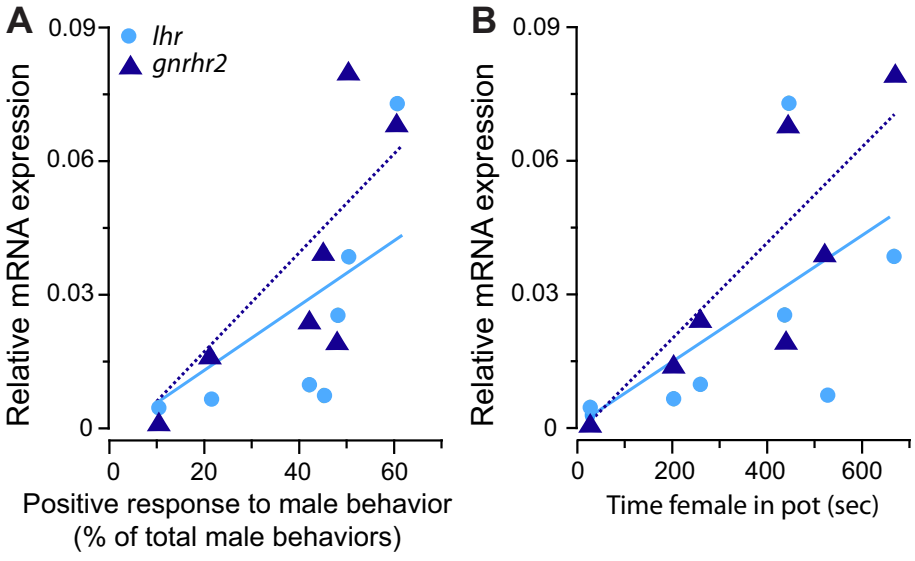

Fig. 5. *gnrhr2* and *lhr* expression in the eye positively correlate with female affiliative behaviors. Expression of gonadotropin system receptors (*lhr*, circle; *gnrhr2*, triangle) positively correlates with female affiliative behaviors (positive response to male behaviors and time in pot). No other neuromodulatory gene expression correlated with female behaviors. N = 7 for all groups.

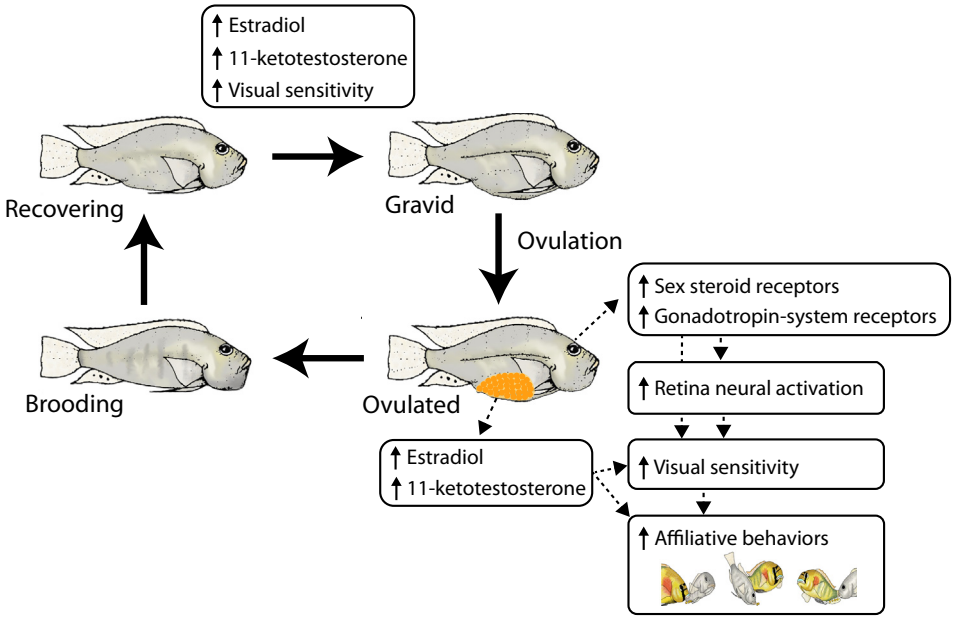

Fig. 6. Summary of reproductive-mediated changes in hormones, behaviors, and visual capabilities of female *A. burtoni*. As females progress through their reproductive cycle towards gravid (i.e. possessing large, ready-to-spawn eggs), levels of circulating steroids increase and visual sensitivity improves. After ovulation, females have an increase in circulating steroid levels, expression of neuromodulatory receptors in the eye, retina neural activity, visual sensitivity, and affiliative behaviors. Although the exact mechanisms are not known, together, this promotes mate choice and spawning.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.yhbeh.2019.06.003.

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