General and Comparative Endocrinology 246 (2017) 200–210

Contents lists available at ScienceDirect

General and Comparative Endocrinology

journal homepage: www.elsevier.com/locate/ygcen

Research paper

Differential sensitivity to estrogen-induced opsin expression in two poeciliid freshwater fish species

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ARTICLE INFO

Article history:
Received 6 August 2016
Revised 29 November 2016
Accepted 19 December 2016
Available online 21 December 2016

Keywords:
Gambusia affinis
Poecilia latipinna
Visual physiology
Retina
Sex steroids
Hormonal modulation

ABSTRACT

The sensory system shapes an individual's perception of the world, including social interactions with conspecifics, habitat selection, predator detection, and foraging behavior. Sensory signaling can be modulated by steroid hormones, making these processes particularly vulnerable to environmental perturbations. Here we examine the influence of exogenous estrogen manipulation on the visual physiology of female western mosquitofish (*Gambusia affinis*) and sailfin mollies (*Poecilia latipinna*), two poeciliid species that inhabit freshwater environments across the southern United States. We conducted two experiments to address this aim. First, we exposed females from both species to a one-week dose response experiment with three treatments of waterborne β-estradiol. Next, we conducted a one-week estrogen manipulation experiment with a waterborne estrogen (β-Estradiol), a selective estrogen receptor modulator (tamoxifen), or combination estrogen and tamoxifen treatment. We used quantitative PCR (qPCR) to examine the expression of cone opsins (SWS1, SWS2b, SWS2a, Rh2, LWS), rhodopsin (Rh1), and steroid receptor genes (ARα, ARβ, ERα, ERβ2, GPER) in the eyes of individual females from each species. Results from the dose response experiment revealed estradiol-sensitivity in opsin (SWS2a, Rh2, Rh1) and androgen receptor (ARα, ARβ) gene expression in mosquitofish females, but not sailfins. Meanwhile, our estrogen receptor modulation experiments revealed estrogen sensitivity in LWS opsin expression in both species, along with sensitivity in SWS1, SWS2b, and Rh2 opsins in mosquitofish. Comparisons of control females across experiments reveal species-level differences in opsin expression, with mosquitofish retinas dominated by short-wavelength sensitive opsins (SWS2b) and sailfins retinas dominated by medium- and long-wavelength sensitive opsins (Rh2 and LWS). Our research suggests that variation in exogenous levels of sex hormones within freshwater environments can modify the visual physiology of fishes in a species-specific manner.

1. Introduction

Increasing evidence suggests that sex steroids may act on sensory systems to influence perception (Remage-Healey, 2012; Forlano et al., 2015). Understanding the interaction between hormones and sensory systems is important, particularly in aquatic ecosystems where anthropogenic changes expose organisms to exogenous hormones and hormone-mimicking substances (Bergman et al., 2013). While hormone modulators in our water sources are known to interfere with reproductive processes at the level of gonadal development (Crews and McLachlan, 2006; Guillette, 2006; Vandenberg et al., 2012), relatively little attention has been given to the effect of these substances on other physiological processes. Research across vertebrates demonstrates that sex steroids are capable of modifying signals and signal processing at the sensory periphery. The effect of endogenous sex steroids on sensory signals and reception has been thoroughly studied in the auditory realm, with ample demonstrations that increased sex steroids can directly affect male call or song rate in birds (Lehrman, 1965; Remage-Healey and Bass, 2010; Remage-Healey, 2012; Remage-Healey et al., 2012, 2013), frogs (Yovanof and Feng, 1983; Lynch and Wilczynski, 2006; Remage-Healey, 2012), and fishes (Remage-Healey and Bass, 2004; Bass, 2008; Remage-Healey, 2012). It is similarly well established that sex steroids influence auditory (Yovanof and Feng, 1983; Sisneros and Bass, 2003; Sisneros et al., 2004; Lynch et al., 2006; Bass, 2008; Forlano et al., 2010) and olfactory (Cardwell et al., 1995; Murphy and Stacey, 2002; Bakker, 2003) processing. In comparison, little is known about how sex steroids directly modify visual sensitivity. In this study, we begin to explore how exogenous hormones influence visual physiology.

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http://dx.doi.org/10.1016/j.ygcen.2016.12.009
0016-6480/

Our current understanding of the interaction between endocrine and visual systems suggests that hormones are likely to be important modulators of visual sensitivity. Behavioral studies with tungara frogs (Cummings et al., 2008), goldfish (Thompson et al., 2004), and sticklebacks (Boulcott and Braithwaite, 2007) demonstrate increased visual sensitivity to mating cues during the reproductive season. The presence of androgen and estrogen receptors along with high levels of aromatase in the fish retina and sensory processing regions of the brain (Gelinas and Callard, 1997; Cotter et al., 2013) provide a possible mechanism by which sex hormones may influence sensory perception. This proposed mechanism of endocrine-dependent visual sensitivity mediated by steroid hormone receptors has gained support from a recent study in sticklebacks that demonstrated increased expression of long-wave-sensitive (LWS) opsin genes in the retina of androgen-implanted males (Shao et al., 2014).

The potential for endogenous, and presumably environmental, sex hormones to modulate the visual system provides yet another mechanism through which the sensory system maintains plasticity during processes of sexual selection. Here we examine the influence of estradiol on female visual sensitivity using two North American poeciliid fish species, the western mosquitofish (*Gambusia affinis*) and sailfin mollies (*Poecilia latipinna*). Poeciliids are particularly well suited for our research questions given that they are widely distributed across gradients of disturbance in the southeastern United States (Kolpin et al., 2002; Heubel, 2004). Furthermore, there is a well documented link between female visual sensitivities and preferences for male ornamentation in some taxa (Rodd et al., 2002; Cummings et al., 2003; Calabrese et al., 2014; Sandkam et al., 2015a), as well as variation in male secondary sexual traits (color ornamentation and behavioral tactics) that is influenced by hormones (Toft and Baatrup, 2001, 2003; Larsson et al., 2002).

The aim of the present study was to investigate how waterborne exposure to estrogen influences visual sensitivity in female mosquitofish (*G. affinis*) and sailfin mollies (*P. latipinna*). We addressed this aim with two experiments. First, we conducted a dose response experiment with both species to compare expression of opsin and steroid receptor genes in eyes taken from individuals exposed to an ethanol control or one of three environmentally relevant doses of estradiol during a one-week waterborne exposure paradigm. Next, we conducted an estrogen manipulation experiment to compare expression of opsin and steroid receptor genes in eyes collected from individuals exposed to an ethanol control, or a treatment of estrogen (β-estradiol), a selective estrogen receptor modulator (tamoxifen), or a combination of β-estradiol and tamoxifen during a one-week waterborne exposure paradigm. To investigate the influence of estrogen on female visual physiology, we used qPCR to examine the expression of rhodopsin (Rh1), and five cone opsins: short wavelength sensitive 1 (SWS1, UV), short wavelength sensitive 2 (SWS2a, SWS2b, blue/violet), medium wavelength sensitive or rod opsin like (Rh2, green), and long wavelength sensitive (LWS, red/orange). To investigate possible mechanisms of estrogen on visual physiology, we also examined the expression of six sex steroid receptors: androgen receptor α (ARα), androgen receptor β (ARβ), estrogen receptor α (ERα/Esr1), estrogen receptor β2 (ERβ2/Esr2b), and G protein estrogen receptor-1 (GPER).

2. Materials and methods

2.1. Estrogen treatment and manipulation

Animals were housed and treated in accordance with the University of Texas at Austin's Institutional Animal Care and Use Committee requirements (protocol AUP-2013-00156). Wild adult mosquitofish (*G. affinis*) females were obtained from San Marcos River in San Marcos, Texas or obtained from semi-wild populations held at the Brackenridge Field Laboratories (University of Texas). Wild adult sailfin molly (*P. latipinna*) females were wild-caught from the San Antonio River in San Antonio, Texas. Once brought into the lab, all females were kept isolated in treatment tanks (n = 10 mosquitofish/tank, n = 5 sailfin mollies/tank) for an acclimation period of at least 2 weeks before experiments began. Day length was kept constant at 12:12 h light:dark and fish were fed daily with Tetramin fish flakes and New Life Spectrum pellets.

We conducted two experiments on non-ovariectomized mosquitofish and sailfin mollies that: i) exposed females to three doses of exogenous estrogen (June 2015), and ii) exposed females to exogenous estrogen and/or an estrogen receptor modulator (Nov 2015). In experiment 1, we conducted a dose response experiment to expose female mosquitofish (MSQ) and sailfins (SFN) to an ethanol vehicle (control) or one of three environmentally relevant (Kolpin et al., 2002) doses of β-estradiol (Sigma Aldrich, St. Louis, MO, USA) for one week. The ethanol control (<1% ethanol; n = 10 MSQ, n = 8 SFN) reflected the maximum amount of ethanol carrier in any treatment tank. The low treatment (15 ng/L E_2; n = 9 MSQ, n = 6 SFN) represented natural US water levels with minimal exposure to estrogen (Kolpin et al., 2002), the medium treatment (150 ng/L E_2; n = 10 MSQ, n = 7 SFN) represented the median level of estrogen measured in moderately disturbed US eastern and upper Midwest streams (Kolpin et al., 2002), and the high treatment (1500 ng/L E_2; n = 10 MSQ, n = 9 SFN) represented concentrations greater than the most highly disturbed sites (Kolpin et al., 2002). In experiment 2, we compared the effects of exogenous β-estradiol and tamoxifen (a selective estrogen receptor modulator) on visual sensitivity by examining opsin and steroid receptor gene expression in eyes collected from individuals exposed to an ethanol control, and either β-estradiol (Sigma Aldrich, St. Louis, MO, USA), tamoxifen (Sigma Aldrich, St. Louis, MO, USA), or a combination of the two in a one-week waterborne exposure paradigm. The ethanol control (<1% ethanol; n = 9 MSQ, n = 6 SFN) reflected the maximum amount of ethanol carrier in any treatment tank. The β-estradiol treatment (150 ng/L E_2; n = 9 MSQ, n = 7 SFN) replicated the medium dosage of E_2 from our previous experiment. Our tamoxifen treatment (50 000 ng/L TAM; n = 6 MSQ, n = 6 SFN) was designed to modulate classic estrogen receptors using a median dosage reported across similar studies of a comparable time frame (Sun et al., 2007; Singh et al., 2012). Tamoxifen is a selective estrogen receptor modulator that can act as an estrogen receptor agonist or antagonist depending on dose and cell/tissue type (Wijayaratne et al., 1999; Menuet et al., 2002; Sabo-Attwood et al., 2007). To the author's knowledge, it is not yet determined whether tamoxifen acts as an estrogen receptor agonist or antagonist in retinal tissue. Therefore, the combination treatment (150 ng/L β-estradiol + 50 000 ng/L TAM; n = 5 MSQ, n = 7 SFN) was included to explore combined effects of exogenous E_2 and tamoxifen-mediated estrogen receptor modulation.

2.2. Gene cloning and qPCR analysis of whole eye expression

To examine the effect of estrogen manipulation, we used qPCR to quantify opsin and steroid receptor mRNA levels. Across vertebrates, the perception of color is mediated by the differential expression of five major classes of opsins: i) rhodopsin (Rh1) and four cone opsins, ii) short wavelength sensitive 1 (SWS1, UV), iii) short wavelength sensitive 2 (SWS2, blue/violet), iv) medium wavelength sensitive or rod opsin like (Rh2, green), and v) long wavelength sensitive (LWS, red/orange) (Yokoyama, 1994, 2000, 2002; Collin et al., 2003). We cloned and then quantified the relative expression of the following genes: rhodopsin (Rh1), cone

opsins (SWS1, SWS2b, SWS2a, Rh2, LWS,), and sex steroid receptors (ARα, ARβ, ERα, ERβ2, GPER). We were unable to isolate ERβ1 transcript from retinal tissue.

For gene cloning, eyes were dissected from 5 to 10 females from each species, incubated overnight at 4 °C in RNAlater (Ambion), and stored at −80 °C until processing. Total RNA was extracted from pooled retinal tissue using Trizol reagent (Invitrogen) following the manufacturer's protocol. cDNA was reverse-transcribed using Invitrogen's First Strand Synthesis kit following the manufacturer's protocol. Cloning primers were designed using MacVector. If the gene sequence had previously been published in Genbank (*G. affinis*: Rh1, ARα, ARβ, ERα, ERβ2; *P. latipinna*: SWS2a, SWS2b, LWS, Rh1, ARα, ARβ, ERα, Gper) we designed gene-specific primers to verify that it was expressed in retinal tissue. For all other genes, we designed degenerate cloning primers derived from published opsin and steroid hormone receptor sequences of closely related poeciliid fish species (Genbank sequence data for *Xiphophorus maculata*, *Poecilia reticulata*, and when available, *Poecilia latipinna*, *Poecilia formosa*, *Gambusia affinis*, *Xiphophorus malinche*, *Xiphophorus birchmanni*). For completeness, we include accession numbers in Table 1 for all of our opsin and steroid hormone receptor sequences whether previously published in Genbank or cloned for this experiment. We have included all gene cloning primer sequences, accession numbers, and clone length in Supplemental Table 1.

For qPCR, females were sacrificed via decapitation, eyes were rapidly dissected and then hemisected, and eye pairs from individual fish in each treatment were collected and placed in RNAlater solution (Ambion) overnight at 4 °C. The RNAlater solution was then removed and eyes were stored at −80 °C until processing. Total RNA was extracted and DNAse-treated using the Maxwell 16 LEV SimplyRNA Tissue Kit (Promega) for 120 eye pairs. For the remaining 14 eye pairs, total RNA was extracted using TRIzol reagent (Invitrogen) according to the manufacturer's protocol, and then DNAse-treated using a turbo DNA-free kit (Ambion). The total RNA from each individual was then reverse-transcribed using Superscript III First-Strand Synthesis SuperMix for qRT-PCR (Invitrogen) in a 40uL-size reaction according to a modified manufacturer's protocol for transcribing cDNA to be used as qPCR template. cDNA synthesis was primed with both oligo-dT and random hexamers. Following the reverse transcription reaction, cDNA was purified using Amicon Ultracentrifugal filters (Millipore) according to the manufacturer's protocol. Real-time qPCR reactions were conducted with SYBR green detection chemistry using a 7900HT real-time PCR machine (ABI) with the following reaction recipe: 10 μL total volume reaction containing 1 μL cDNA template, 5 μL 2× POWER SYBR Green PCR master mix (ABI), and 5 pmol primer. Real-time primer sequences, amplicon lengths, accession number, and PCR efficiencies are reported in Table 1. PCR conditions were as follows: 95 °C for 10:00 followed by 40 cycles of 95 °C for 00:15, 60 °C for 00:30, and 72 °C for 00:30. Each sample was run in triplicate.

Results were first analyzed using Applied Biosystems Sequence Detection Software (SDS vs. 2,3), and gene expression levels were normalized by deriving the residuals from a linear regression of the measured gene concentration (based on standard curve estimates and averaged across triplicates) by input cDNA concentration, as in and Lynch et al. (2012), Ramsey et al. (2012, 2014), and Wang et al. (2014). Input cDNA concentration was measured using Quanti-iT RiboGreen RNA reagent (Molecular Probes) using a modified version of the manufacturer's protocol that measured input single-stranded cDNA rather than input RNA (as in Cummings et al., 2008; Lynch et al., 2012; Ramsey et al., 2014; Wang et al., 2014). Using Ribogreen to measure cDNA has been previously verified in our lab (Cummings et al., 2008). In all other respects we followed the manufacturer's protocol. The derived residuals represent the deviation of the observed gene cDNA quantity from the predicted cDNA quantity (based on cDNA input), and can be used to infer normalized gene expression levels, as in Lynch et al. (2012) and Ramsey et al. (2012, 2014).

Proportional cone opsin expression was determined as a fraction of the total cone opsin genes expressed for an individual (Carleton and Kocher 2001) according to:

$$\frac{T_i}{T_{\text{all}}} = \frac{\left(1/\left((1+E_i)^{C,t_i}\right)\right)}{\sum\left(1/\left((1+E_i)^{C,t_i}\right)\right)}$$

where T_i/T_{all} is the relative gene expression ratio for a given gene normalized by the total cone opsin genes expressed, E_i is the PCR efficiency for a given gene, and Ct_i is the cycle threshold number for a given gene. To calculate the PCR efficiency for a given gene, critical cycle number was measured for standard curve of 6 serial dilutions of cDNA covering a 100000-fold range. PCR efficiency (E_i) was then determined from the slope of a plot of ln(concentration) versus critical cycle number such that $E_i = 10^{(1/\text{slope}_i)}$. The PCR efficiency for a given gene in each species is provided in Table 1.

2.3. Data analysis

All analyses were carried out using R version 3.2.4. For each experiment, statistical analyses were carried out on two measures of gene expression i) gene residuals calculated from raw qPCR and ribogreen values, and ii) proportional cone opsin data. Gene residuals provide an indication of which genes are differentially regulated by estrogen manipulation, while proportional measures of opsin expression represent a ratio of different retinal cone cell types (Fuller et al., 2004) to provide a measure of possible color vision differences based on estrogen manipulation.

For all analyses, we removed samples subject to technical error (e.g. extreme ratios of gene expression: cDNA input). For experiment 1, we removed 5 mosquitofish samples (control: n = 1, low E_2: n = 2, medium E_2: n = 2) and 3 sailfin samples (low E_2: n = 1, medium E_2: n = 2). For experiment 2, we removed 4 mosquitofish samples (control: n = 2, low tamoxifen: n = 2). All analyses were carried out on this edited data set for consistency since it yielded identical patterns to analyses with the full data set (data not shown).

For experiment 1, five of the eleven gene residuals were normally distributed in mosquitofish (n = 5: Rh1, Rh2, SWS2b, ARβ, GPER) and only one in sailfins (n = 1: Rh1). In contrast, four of the five cone opsin proportions were normally distributed in both mosquitofish (n = 4: LWS, Rh2, SWS2a, SW2b) and sailfins (n = 4: LWS, Rh2, SWS2a, SWS1). For experiment 2, the residuals of three of the eleven genes were normally distributed in mosquitofish (n = 3: ARα, ERα, ERβ) and four genes in sailfins (n = 4: ARα, ERα, ERβ, GPER). For our proportional analyses, only one of five cone opsin proportions were normally distributed in mosquitofish (n = 1: LWS), whereas four of five opsin proportions were normally distributed in sailfins (n = 4: LWS, Rh2, SWS2a, SWS2). Tests using untransformed or $\log_{10}$-transformed values yielded identical patterns. Similarly, non-parametric Kruskal-Wallis tests yielded nearly identical patterns to ANOVA tests for these gene residuals and proportions (data not shown), so only MANOVA and ANOVA results will be reported in the text for consistency.

For each experiment, multivariate analyses were first carried out using MANOVA to explore the effects of treatment and species on either gene residuals or cone opsin proportion. Group-wise comparisons of individual gene residuals or cone opsin proportions were then measured with ANOVA tests to explore effects of treatment, followed by Tukey's test to correct for multiple comparisons. Group-wise comparisons exploring the effect of experiment on

Table 1
Real-time qPCR primers and reaction parameters.

Species	Gene	Accession Number	Amplicon Length	PCR efficiency	Primer sequence
G. affinis	SWS1	KX384593	87	2.162	For: 5′-CCGACTGGTACACCCACAAT-3′ Rev:5′-TGGACAGAGGCATGCAGAAG-3′
	SWS2a	KX384594	74	2.321	For: 5′-TCTGCTTCTGCTTTGCCGTA-3′ Rev: 5′-TGGTCACCTCCTTTTCAGCC-3′
	SWS2b	KX384595	144	2.077	For: 5′-TTATCTGCAAGCCACTCGGG-3′ Rev: 5′-AACACTGCATTCCCTCAGGG-3′
	Rh2	KX384592	86	2.022	For: 5′-TCAGAACAAAAAGCTGCGCC-3′ Rev: 5′-TGAAACCAAAGGCGCACATG-3′
	LWS	KX384591	133	1.960	For: 5′-TTCAGTGGAAGCGAAGACCC-3′ Rev: 5′-GCATAGCAACAGCACGGATG-3′
	Rh1	Y11146.1	90	1.968	For: 5′-CGGTGAAATCGGTCTCTGGT-3′ Rev: 5′-TCAGTGAAGCGGAAGTTGCT-3′
	ARα	AB182328.1	125	2.146	For: 5′-GTTGTCATGGATGGGGGTGA-3′ Rev: 5′-TGCTGGACACTTGCATACGT-3′
	ARβ	AB182329.1	121	2.399	For: 5′-ATACCAGGTTTGAGGCTGGC-3′ Rev: 5′-CATGTGAGCGCACCGTAATG-3′
	ERα	AB295655.1	135	2.815	For: 5′-TGGCGGAGATCTTCGACATG-3′ Rev: 5′-TCGTTCCGGTGCAGAAAGAA-3′
	ERβ2	AB295657.1	118	2.778	For: 5′-CAGTGTGTGGAGGGCATCAT-3′ Rev: 5′-TGAGCAGGATCATGGCCTTG-3′
	GPER	KX384596	102	2.218	For: 5′-CGTCCTCTGCACCTTCATGT-3′ Rev: 5′-CTGGCCAAGGCGATGTATCT-3′
P. latipinna	SWS1	KX384599	87	2.064	For: 5′-CCGACTGGTACACCCACAAT-3′ Rev:5′-TGGACAGAGGCATGCAGAAG-3′
	SWS2a	XM_015060723.1	74	2.508	For: 5′-TCTTTGGCAGTGGTAGCGTT-3′ Rev: 5′-AACTGAGGCTGTCAATGCGA-3′
	SWS2b	XM_015060722.1	144	2.083	For: 5′-TTATCTGCAAGCCACTCGGG-3′ Rev: 5′-AACACTGCATTCCCTCAGGG-3′
	Rh2	KX384598	148	1.924	For: 5′-TGCTCCTGTGGACCTGACTA-3′ Rev: 5′-CGGCAGCTTTGACTGTCATG-3′
	LWS	XM_015048685.1	133	2.030	For: 5′-TTCAGTGGAAGCGATGACCC-3′ Rev: 5′-GCATAGCAACAGCACGGATG-3′
	Rh1	XM_015047258.1	109	2.137	For: 5′-CATCAGCAACTTCCGCTTCG-3′ Rev: 5′-GTAACGAGACCAGCCGACAA-3′
	ARα	KP172522.1	125	2.009	For: 5′-GTTGTCATGGATGGGGGTGA-3′ Rev: 5′-TGCTGGACACTTGCATACGT-3′
	ARβ	KP172525.1	121	2.603	For: 5′-ATACCAGGTTTGAGGCTGGC-3′ Rev: 5′-CATGTGAGCGCACCGTAATG-3′
	ERα	XM_015031281.1	120	2.897	For: 5′-CATGGCGGAGATCTTCGACA-3′ Rev: 5′-GAAAACGCGCCAGAGTTGAG-3′
	ERβ2	KX384601	118	3.016	For: 5′-CAGTGTGTGGAGGGCATCAT-3′ Rev: 5′-TGAGCAGGATCATGGCCTTG-3′
	GPER	XM_015059316.1	102	2.394	For: 5′-CGTCCTCTGCACCTTCATGT-3′ Rev: 5′-CTGGCCAAGGCGATGTATCT-3′

individual cone opsin proportions were carried out for each species using ANOVA and Tukey's test.

3. Results

3.1. Effects of estrogen manipulation on gene residuals

In the first experiment, multivariate analyses using MANOVA on opsin residuals from a combined dataset with both species (mosquitofish and sailfin mollies) revealed an effect of treatment (Pillai's trace = 0.678, $F = 2.434$, df = (3,53), $p < 0.01$), and an effect of the interaction between species and treatment (Pillai's trace = 0.506, $F = 1.692$, df = (3,53), $p < 0.05$). Follow up analyses using ANOVA with post-hoc Tukey corrections for multiple comparisons revealed that a dose-dependent effect of E_2 on Rh1, SWS2a, and Rh2 could only be detected in mosquitofish females (Fig. 1, Supplementary Fig. 1). In female mosquitofish, group-wise comparisons using ANOVA on opsin residuals revealed that Rh1 ($F = 3.58$, df = (3,30), $p < 0.05$) showed significant differences between low and high treatments; SWS2a showed significant differences between moderate doses of E_2 and all other treatments ($F = 6.29$, df = (3,30), $p < 0.01$); and Rh2 showed significant differences between high and low, as well as high and control treatments ($F = 5.35$, df = (3,30), $p < 0.01$). Multivariate analyses using MANOVA on steroid receptor residuals from a combined dataset with both species (mosquitofish and sailfin mollies) revealed a significant effect of the interaction between species and treatment (Pillai's trace = 0.491, $F = 1.84$, df = (3,49), $p < 0.05$). Follow up analyses using ANOVA with post-hoc Tukey corrections for multiple comparisons revealed that a dose-dependent effect of E_2 on ARα and ARβ could only be detected in mosquitofish females (Fig. 1). In female mosquitofish, group-wise comparisons using ANOVA on steroid receptor residuals revealed that ARα ($F = 4.24$, df = (3,30), $p < 0.05$) showed significant differences between low and medium treatments; ARβ ($F = 5.63$, df = (3,30), $p < 0.01$) showed significant differences between low and medium treatments, as well as low and high treatments.

In the second experiment, multivariate analyses using MANOVA on opsin residuals from a combined dataset of estrogen manipulation in both species revealed an effect of treatment (Pillai's trace = 0.936, $F = 3.022$, df = (3,43), $p < 0.001$), and an effect of the interaction between species and treatment (Pillai's trace = 0.677, $F = 1.94$, df = (3,43), $p < 0.05$). Follow up analyses using ANOVA with post-hoc Tukey corrections for multiple comparisons revealed that the effect of estrogen manipulation on SWS1, SWS2b, and Rh2 could only be detected in mosquitofish females, while the effect of estrogen manipulation on LWS was significant in both species (Fig. 2, Supplementary Fig. 2). In female mosquitofish,

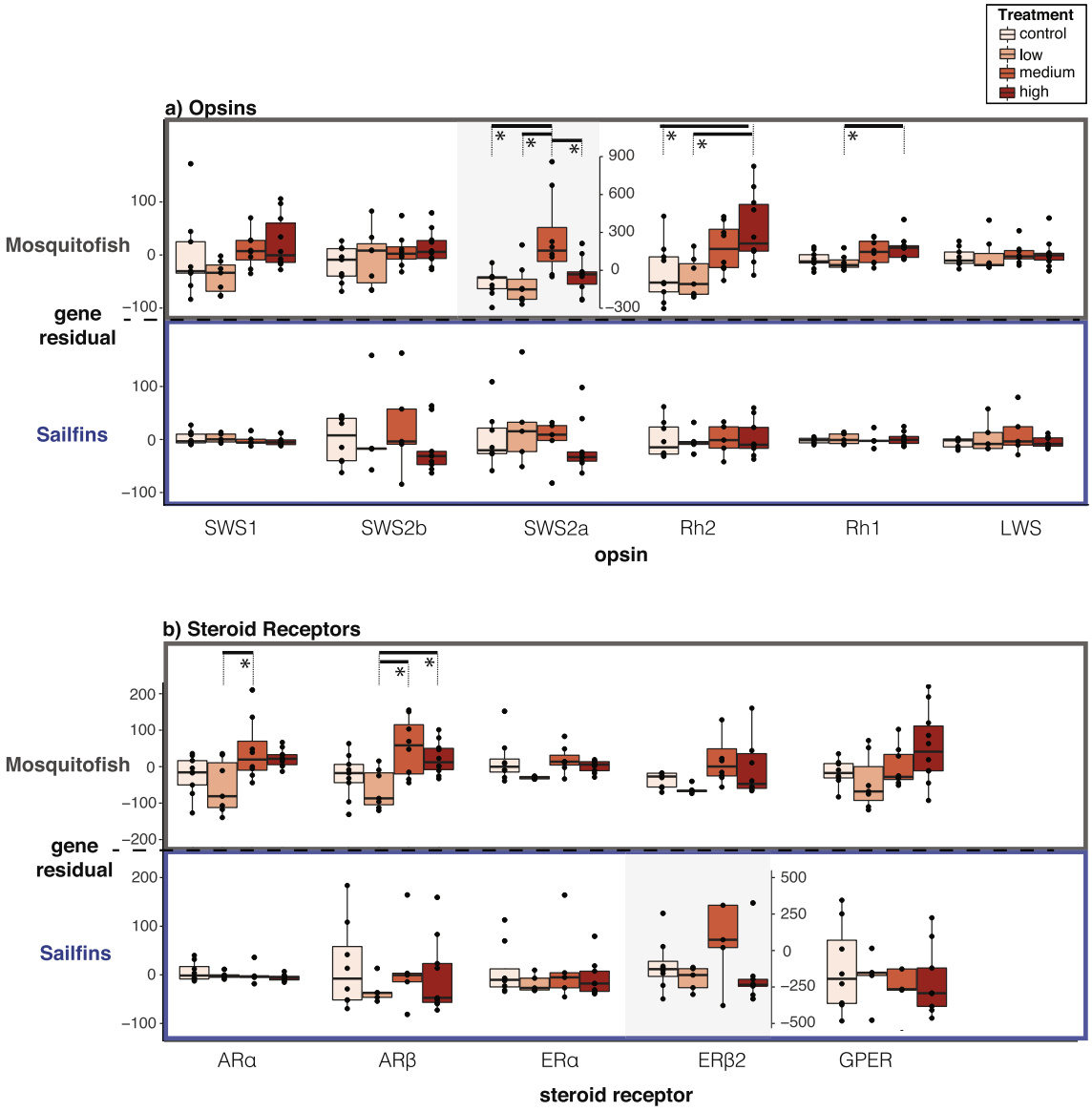

Fig. 1. Boxplot of (a) opsin and (b) steroid receptor gene residuals in retinal tissue from female mosquitofish (MSQ) and sailfin mollies (SFN) exposed to an E_2 dose response study. The treatments include an ethanol control (<1% ethanol; n = 9 MSQ, n = 8 SFN), low E_2 (15 ng/L E_2; n = 7 MSQ, n = 5 SFN), medium E_2 (150 ng/L E_2; n = 8 MSQ, n = 5 SFN), and high E_2 treatment (1500 ng/L E_2; n = 10 MSQ, n = 9 SFN). Relative opsin and steroid receptor mRNA levels were quantified using qPCR, gene expression levels were normalized by deriving the residuals from a linear regression of the measured gene concentration by input cDNA. To provide a common scale for our expression patterns, y-axis values reflect normalized residuals of all six opsins (a) or all five steroid receptors (b) except for genes requiring a separate scale bar (highlighted in grey). In those cases, a separate scale bar is provided on the right (for MSQ: SWS2a, for SFN: ERβ2). To provide a common scale for our expression patterns across multiple genes, y-axis values were bounded such that 6 data points were obstructed from view (MSQ steroids: ERβ2 (1 control, 1 medium, 1 high); SFN steroids: ERβ2 (1 medium), GPER (2 medium)). These points were included in our statistical analyses and are provided on unbounded boxplots (Supplementary Fig. 1). A horizontal line and asterisk highlights significant differences between treatment groups at the outermost end of the line ($p < 0.05$, ANOVA followed by Tukey's test).

group-wise comparisons using ANOVA on opsin residuals revealed that SWS1 showed significant differences between control and tamoxifen-E_2 treatments ($F = 4.01$, $df = (3,21)$, $p < 0.05$); SWS2b exhibited significant differences between the tamoxifen-E_2 treatment and the control and tamoxifen treatments ($F = 5.52$, $df = (3,21)$, $p < 0.01$); Rh2 exhibited significant differences between tamoxifen and both tamoxifen-E_2 and E_2 treatments ($F = 6.33$, $df = (3,21)$, $p < 0.01$); and LWS exhibited significant differences between estrogen and tamoxifen treatments ($F = 4.48$, $df=(3,21)$, $p < 0.05$).

In female sailfins, group-wise comparisons of opsins revealed that LWS exhibited significant differences between estrogen and tamoxifen treatments ($F = 4.83$, $df = (3,22)$, $p < 0.01$). Multivariate analyses using MANOVA on steroid receptor residuals from a combined dataset with both species (mosquitofish and sailfin mollies) revealed no significant effect of treatment (Pillai's trace = 0.269, $F = 0.809$, $df = (3,43)$, $p = 0.66$).

3.2. Measuring changes in proportional cone opsin expression

In experiment one, there was no effect of estradiol treatment on cone opsin proportions. However, one-way ANOVAs revealed a significant difference in the cone opsin proportions in unmanipulated controls between the two species (Fig. 3, Supplementary Fig. 3). With the exception of SWS2a, all of the cone opsins were significantly different between the unmanipulated control groups of the two species; this includes SWS1 ($F = 15.861$, $df = (1,15)$, $p < 0.01$), SWS2b ($F = 901.55$, $df = (1,15)$, $p < 0.001$), Rh2

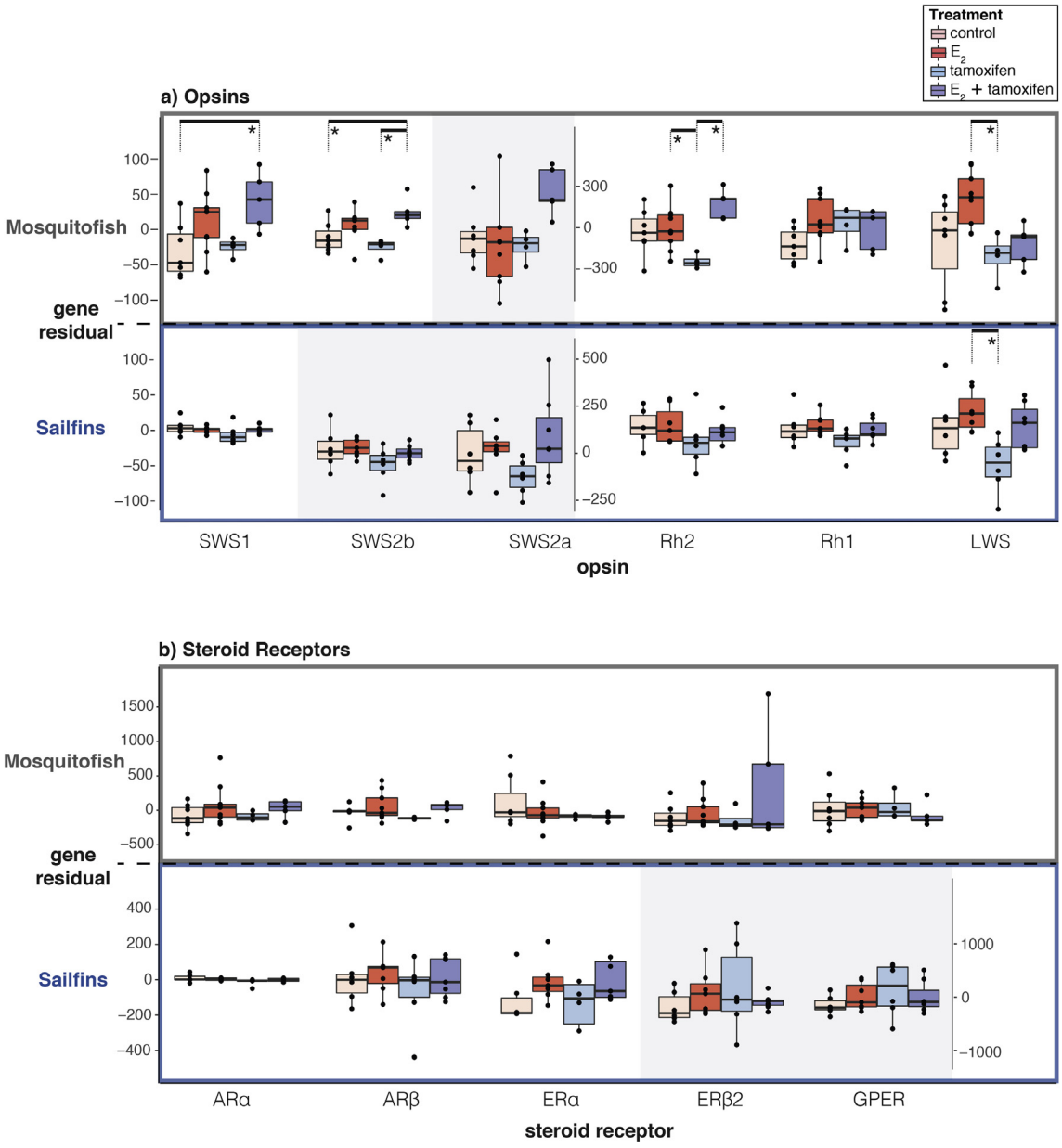

Fig. 2. Boxplot of (a) opsin and (b) steroid receptor gene residuals in retinal tissue from female mosquitofish (MSQ) and sailfin mollies (SFN) exposed to an E_2 manipulation study. The treatments include an ethanol control (<1% ethanol; n = 7 MSQ, n = 6 SFN), E_2 treatment (150 ng/L E_2; n = 9 MSQ, n = 7 SFN), tamoxifen treatment (50,000 ng/L TAM; n = 4 MSQ, n = 6 SFN), and combination E_2-tamoxifen treatment (150 ng/L β-estradiol + 50,000 ng/L TAM; n = 5 MSQ, n = 7 SFN). Relative opsin and steroid receptor mRNA levels were quantified using qPCR, and gene expression levels were normalized by deriving the residuals from a linear regression of the measured gene concentration by input cDNA. To provide a common scale for our expression patterns, y-axis values reflect normalized residuals of all six opsins (a) or all five steroid receptors (b) except for genes requiring a separate scale bar (highlighted in grey). In those cases, a separate scale bar is provided on the right (for MSQ: SWS2a, for SFN: SWS2b, SWS2a, ERβ2, GPER). To provide a common scale for our expression patterns across multiple genes, y-axis values were bounded such that 4 data points were obstructed from view (MSQ opsins: SWS2a (1 E_2); SFN steroids: ERα (2 tamoxifen, 1 E_2-tamoxifen)). These points were included in our statistical analyses and are provided on unbounded boxplots (Supplementary Fig. 2). A horizontal line and asterisk highlights significant differences between treatment groups at the outermost end of the line ($p < 0.05$, ANOVA followed by Tukey's test).

($F = 18.483$, $df = (1,15)$, $p < 0.001$), and LWS ($F = 23.804$, $df = (1,15)$, $p < 0.001$). The relative proportion of cone opsins in unmanipulated mosquitofish females, ranking from most to least abundant, was SWS2b (84%) > Rh2 (15%) > LWS (<1%) > SWS1 (<1%) > SWS2a (~0%). The relative proportion of cone opsins in unmanipulated sailfin females, ranking from most to least abundant, was Rh2 (53%) > LWS (26%) > SWS1 (21%) > SWS2b (<1%) > SWS2a (~0%).

In experiment two, there was an effect of treatment on the proportion of one cone opsin in mosquitofish, but not in sailfins. Group-wise comparisons using ANOVA revealed that SWS2b was significantly different between tamoxifen and E_2 treatments ($p < 0.05$) in mosquitofish, but not in sailfins. In species comparisons between unmanipulated controls for the experiment 2 fish, one-way ANOVAs revealed a significant difference in the cone opsin proportions in unmanipulated controls between the two species (Fig. 3). With the exception of SWS2a, all of the cone opsins were significantly different between the two species; this includes SWS1 ($F = 127.09$, $df = (1,11)$, $p < 0.001$), SWS2b ($F = 846.87$, $df = (1,11)$, $p < 0.001$), Rh2 ($F = 7.04$, $df = (1,11)$, $p < 0.05$), and LWS

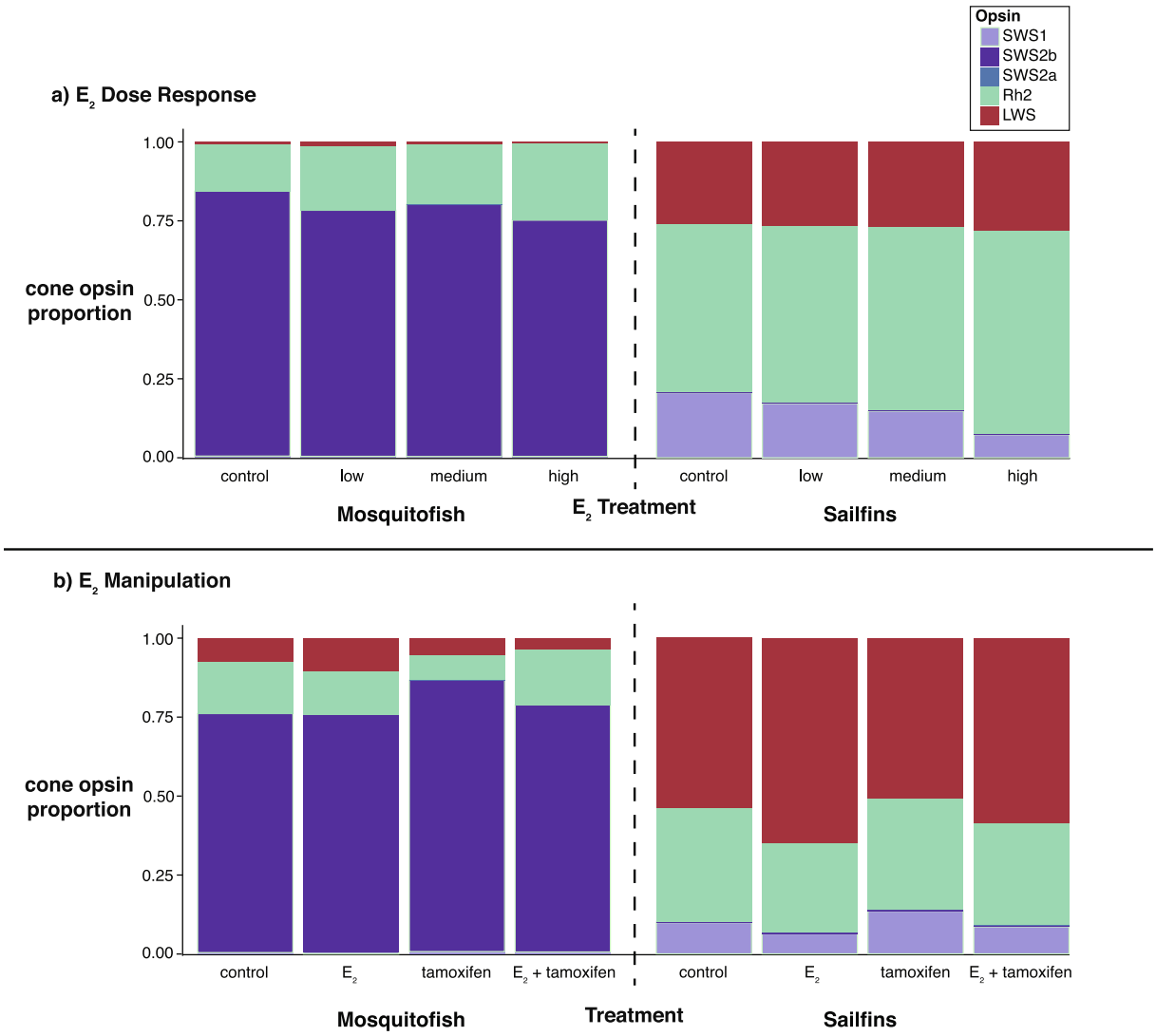

Fig. 3. Stacked bar plot of the proportional expression of cone opsins in retinal tissue from female mosquitofish (MSQ) and sailfin mollies (SFN) exposed to an a) E_2 dose response, and b) E_2 manipulation study. Proportional cone opsin expression was determined as a fraction of the total cone opsin genes expressed for an individual.

($F = 41.64$, $df = (1,11)$, $p < 0.001$). The relative proportion of cone opsins in unmanipulated mosquitofish females, ranking from most to least abundant, was SWS2b (75%) > Rh2 (16%) > LWS (7%) > SWS1 (<1%) > SWS2a (~0%). The relative proportion of cone opsins in unmanipulated sailfin females, ranking from most to least abundant, was LWS (54%) > Rh2 (36%) > SWS1 (10%) > SWS2b (<1%) > SWS2a (~0%).

One-way ANOVA revealed a significant difference in cone opsin proportions in unmanipulated controls for each species between experiments (cone opsin proportions in Experiment 1 vs. Experiment 2) as well. In mosquitofish, one-way ANOVA revealed experimental differences in the relative expression of SWS2b ($F = 5.11$, $df = (1,14)$, $p < 0.05$), SWS2a ($F = 13.84$, $df = (1,14)$, $p < 0.01$), and LWS ($F = 16.68$, $df = (1,14)$, $p < 0.01$). In sailfins, one-way ANOVA revealed experimental differences in the relative expression of LWS ($F = 9.50$, $df = (1,12)$, $p < 0.01$). Welch's *t*-test revealed a significant increase ($t = -8.667$, $df = 55.66$, $p < 0.001$) in mosquitofish size (measured in total length) during the second experiment (TL = 43.12 mm) relative to the first experiment (TL = 34.00 mm), but no difference ($t = 0.428$, $df = 48.33$, $p = 0.67$) in sailfin size between experiment 2 (TL = 40.38 mm) and experiment 1 (TL = 41.37 mm).

4. Discussion

4.1. Species-specific effects of estrogen manipulation on differential gene regulation

In the current study, we detected estrogen-induced changes in the visual physiology of females from two poeciliid species, western mosquitofish and sailfin mollies, across doses of E_2 that are environmentally relevant (Kolpin et al., 2002). The results of our β-estradiol dose response experiment demonstrated increased opsin and androgen receptor gene expression associated with increasing doses of E_2 in mosquitofish, but not in sailfins. Our estrogen manipulation experiment suggests that tamoxifen modulates estrogen receptors in a complex cell-specific manner in retinal tissue, possibly acting as an estrogen receptor antagonist in photoreceptors containing specific opsins. In both species, LWS opsin gene expression was significantly decreased in tamoxifen relative to E_2 treatments. In mosquitofish, SWS2b and Rh2 gene expression was significantly decreased in tamoxifen relative to combination E_2-tamoxifen treatments, and SWS1 and SWS2b gene expression was significantly increased in combination E_2-tamoxifen treatments relative to controls. However, these results

should be viewed with caution as our study employed non-ovariectimized wild-caught females with unknown circulating levels of (or prior exposure to) estradiol.

The results of our experiment extend what was previously known about hormone-mediated changes in the visual physiology of fishes. Earlier reports established that exogenous exposure to thyroid hormone results in differential expression of opsin genes in coho salmon (Temple et al., 2008), and that administration of the androgen 11-KT results in upregulated long-wave sensitive opsin genes in three-spine sticklebacks (Shao et al., 2014). To the best of our knowledge, our research is the first to manipulate estrogen signaling and subsequently examine both opsin and sex steroid receptor gene expression in female retinas.

Our current findings suggest that increases in opsins due to exogenous steroid manipulation is mediated through estrogen signaling without a concomitant change in estrogen receptor transcript levels (Figs. 1 and 2), but that estrogen receptors may be part of a signaling cascade that can regulate opsin expression within the retina. Although we were unable to detect ERb1 expression in teleost retina tissue in the current study, we detected retinal expression of three estrogen (ERα, ERβ2, and GPER) and two androgen (ARα, ARβ) receptors in low but measurable amounts. This includes estrogen receptor types that act primarily as nuclear transcription factors (ERα, ERβ2) and a transmembrane G protein-coupled receptor (GPER) that acts primarily through non-genomic actions (Thomas et al., 2005; Prossnitz and Barton, 2011). In both experiments, the steroid receptor that was most proportionally abundant in each species ($\geqslant$99%) was ARα. This may suggest that androgen receptors play a role in hormone-mediated modulation of the visual system since gene expression levels of both ARα and ARβ were significantly affected by E_2 treatment in mosquitofish. Considering recent evidence demonstrating that androgen administration regulates opsin expression in male sticklebacks (Shao et al., 2014), our data may suggest a species-specific role for androgen receptors in the modulation of visual physiology. Interestingly, when considering only the expression of estrogen receptors (ERα, ERβ2, and GPER), GPER was the most proportionally abundant ($\geqslant$99%) across both experiments in each species. No studies to date have investigated the role of GPER in hormone-mediated tuning of sensory systems.

To further explore potential mechanisms of hormone-mediated changes in visual physiology, we exposed females to tamoxifen since it acts as a selective estrogen receptor modulator with tissue specific effects (Wijayaratne et al., 1999). Tamoxifen modulates estrogen signaling by competitively binding to the AF2 transcriptional activation domain of ERα and ERβ receptors, with mixed effects on GPER (Bardet et al., 2002; Menuet et al., 2002; Sabo-Attwood et al., 2007). Prior to this experiment, the effects of tamoxifen on retinal tissue were unknown. While our tamoxifin treatment did not significantly alter gene expression of any steroid receptors in either species, when tamoxifen treatments were compared against E_2 treatments, tamoxifen significantly decreases Rh2 opsin gene expression in mosquitofish, and significantly decrease LWS opsin gene expression in both species. This could suggest that tamoxifen acts antagonistically on ERα and ERβ2 in the eyes of these fishes, and further supports species-specificity in the role of the estrogen receptors in modulating opsin expression in the retina.

The species-specific effects observed in our study are particularly intriguing. Mosquitofish females exhibited estrogen-dependent gene expression in multiple opsins (LWS, RH2, SWS2b, SWS1) and steroid receptors (ARα, ARβ), whereas sailfins only exhibited estrogen-dependent modulation of the LWS opsin. Previous research into estrogen-dependent sensory tuning (e.g. midshipman auditory tuning: Bass, 2008; tungara frog visual responsiveness: Cummings et al., 2008; tungara frog auditory responsiveness: Lynch and Wilczynski, 2006) has occurred in species in which females discriminate amongst male signaling or courtship properties (e.g. calls, ornaments). In the present study, mosquitofish represent a species in which male coercion predominates, while sailfin mollies represent a species in which females discriminate amongst ornamented males (Plath et al., 2007; Fraser et al., 2014). Our findings of greater sensory responsiveness to exogenous steroids in mosquitofish relative to sailfin females is consistent with previous studies that have demonstrated that the magnitude of sex steroid mediated plasticity in fish can vary with mating system (Hirschenhauser et al., 2004; Oliveira, 2009). Under this framework, one possible explanation for the increased estrogen sensitivity of opsin gene expression in mosquitofish females may be increased pressure to avoid sexual harassment during the breeding season. Previous studies with western mosquitofish have revealed that individuals display robust preferences for animated images of conspecifics with enhanced yellow pigmentation (Polverino et al., 2013), and studies with other mosquitofish species (*Gambusia Hurtadoi*: McAlister, 1958) demonstrate that increased social rank is positively correlated with male yellow coloration. Consequently, a female preference for yellow male coloration may reflect an attraction for dominant males and concomitant avoidance of sexual harassment from subordinate males (Pilastro et al., 2003; Dadda et al., 2005) that may be mediated by sex steroid receptors. To explore potential sex differences, future studies will need to extend our analyses to examine effects of exogenous steroid manipulation on male visual physiology as well.

4.2. Changes in proportional cone opsin expression

Proportionally, middle- and short-wavelength sensitive (Rh2, SWS2b, respectively) opsins are most predominant in unmanipulated mosquitofish females, while long- and middle-wavelength sensitive (LWS, Rh2, respectively) opsins are most predominant in unmanipulated sailfin females. As differences in opsin gene expression are expected to be correlated with visual sensitivity (Fuller et al., 2003, 2010; Horth, 2007; Seehausen et al., 2008), we might expect these species-specific differences in opsin gene expression to be related to species-level differences in optical habitat use or visual tasks (Seehausen et al., 2008; Sakai et al., 2016). For instance, previous studies in cichlids, guppies, and bluefin killifish have revealed that fish in clear water habitats express more short-wavelength sensitive cone opsins (SWS2b in cichlids: Hofmann et al., 2009; SWS1 and SWS2b in bluefin killifish: Fuller et al., 2004, 2010; Fuller and Claricoates, 2011), while fish in murky habitats express more long-wavelength sensitive opsins (LWS double cones in cichlids: Carleton et al., 2005; LWS-1, LWS-2, LWS-3, LWS-R in guppies: Ehlman et al., 2015; Rh2 and LWS in bluefin killifish: Fuller et al., 2004, 2010; Fuller and Claricoates, 2011), although these patterns are not always as clear across species (Smith et al., 2011). Previous studies have also demonstrated cone opsin expression to vary with depth both within and across species, with fish from shallow habitats exhibiting shorter wavelength sensitivity (Parry et al., 2005; Stieb et al., 2016). While western mosquitofish and sailfin mollies in Central Texas frequently coexist in the same aquatic habitats, previous studies have demonstrated that the two species exhibit distinct patterns of habitat selection (Chick and Mlvor, 1997). Based on species-specific cone opsin proportions observed in this study, we might expect mosquitofish to inhabit shallow, clear habitats consistent with shorter wavelength sensitivity, and sailfin mollies to inhabit deep, murky habitats consistent with longer wavelength sensitivity.

Interestingly, we observed increased relative expression of long-wavelength sensitive opsin (LWS) in both mosquitofish and sailfins during our second experiment (Nov 2015) relative to our

first experiment (June 2015). This coincided with a significant increase in mosquitofish but not sailfin total length (TL). This is consistent with previous studies that have demonstrated altered opsin expression either as part of development (black bream: Shand et al., 2008) or as a result of changes in the social or physical environment (cichlids: Smith et al., 2011, guppies: Sandkam et al., 2015a; Fuller et al., 2004), although our results suggest these trends may be species-specific.

4.3. Environmental and social implications of estrogen modulated visual plasticity

Our results have important implications for understanding how environmental perturbations, specifically the addition of hormone modulating substances, may influence the physiology and behavior of aquatic organisms. Although the mechanisms underlying, and functional significance of, hormonal modulation of opsin gene expression are still unclear, our findings suggest that species-specific physiological and environmental differences may influence the modulation of the visual sensory system. It is unclear whether there are sex-specific effects of steroids on the visual sensory system, but this is an important topic for future research.

These findings have significant implications for steroid-induced plasticity in visual physiology that may exhibit indirect effects on behavioral interactions such as female mate choice or male-male interactions. Many poeciliid fishes use ornamentation that varies from short- (e.g. UV in northern swordtails: Cummings et al., 2003; UV in sailfin mollies: Palmer and Hankison, 2015) to long-wavelength signals (e.g. orange in guppies: Sandkam et al., 2015a) to communicate with potential mates or rivals. As humans alter the exogenous steroid environment, this is likely to have a direct physiological influence on opsin expression that may result in perceptual changes of signaling cues. This is of particular importance if interactions with conspecifics driven by short- and long-wavelength sensitive visual communication across poeciliids can become confounded by species-specific steroid induced plasticity.

In accordance with previous studies investigating phenotypic plasticity in opsin expression (e.g. Fuller et al., 2005; Hofmann et al., 2010), our experiments relied on quantification of opsin transcripts from whole eyes using qPCR; however, the observed differences in opsin expression in this study might not reflect changes in the abundance of various cone types or fundamental alterations to the color vision of the animal. Although we measured rhodopsin and five cone opsins in this study, previous research has reported some poecilliid species to possess 9 opsin proteins (Watson et al., 2011), including 4 distinct LWS loci: LWS-1, LWS-2, LWS-3, and LWS-R (Sandkam et al., 2013), and 2 distinct RH2 loci: RH2-1, RH2-2 (Hoffmann et al., 2007). Primers in the present study were designed to bind to any and all transcript variants of LWS, and may not have been sensitive to changes in gene expression exhibited by only a limited number of these LWS variants. This is of particular importance since recent studies have found differences in LWS-1 allele frequencies across species of poeciliid to differ (Sandkam et al., 2015b). Future studies incorporating behavioral studies with males and females that assess behavioral sensitivity (e.g. optomotor and optokinetic experiments) along with measures of the visual sensory system's response to estrogen manipulation are needed to extend our findings of steroid-induced plasticity in opsin gene expression to the realm of visual physiology and associated mate choice and male agonistic processes (see Table 1).

5. Conclusion

Over the last fifty years, many investigators have shown that sex hormones can have profound effects on sensory systems, as well as the detection and discrimination of sensory cues involved in mate choice. This study adds to a growing body of evidence suggesting that sex hormones may influence our visual perception of the surrounding world based on their modulation of sensory systems. Further research is needed to explore the functional significance and species-specificity of hormone-mediated alterations to opsin gene expression.

Acknowledgments

The authors thank referees and two anonymous reviewers for helpful comments, and are grateful to members of the Cummings lab for assistance and discussion. This work was made possible by support from an EEB Start-up Grant, IB Recruitment Fellowship, Texas Ecolab Grant, and NSERC Postgraduate Student Grant (PGS) to CNF.

Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.ygcen.2016.12.009.

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