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RESEARCH ARTICLE

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Estrogenic signaling and sociosexual behavior in wild sex-changing bluehead wrasses, *Thalassoma bifasciatum*

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Abstract

Estrogenic signaling is an important focus in studies of gonadal and brain sexual differentiation in fishes and vertebrates generally. This study examined variation in estrogenic signaling (1) across three sexual phenotypes (female, female-mimic initial phase [IP] male, and terminal phase [TP] male), (2) during socially-controlled female-to-male sex change, and (3) during tidally-driven spawning cycles in the protogynous bluehead wrasse (*Thalassoma bifasciatum*). We analyzed relative abundances of messenger RNAs (mRNAs) for the brain form of aromatase (*cyp19a1b*) and the three nuclear estrogen receptors (ER) (*ERα*, *ERβa*, and *ERβb*) by qPCR. Consistent with previous reports, forebrain/midbrain *cyp19a1b* was highest in females, significantly lower in TP males, and lowest in IP males. By contrast, *ERα* and *ERβb* mRNA abundances were highest in TP males and increased during sex change. *ERβa* mRNA did not vary significantly. Across the tidally-driven spawning cycle, *cyp19a1b* abundances were higher in females than TP males. Interestingly, *cyp19a1b* levels were higher in TP males close (~1 h) to the daily spawning period when sexual and aggressive behaviors rise than males far from spawning (~10–12 h). Together with earlier findings, our results suggest alterations in neural estrogen signaling are key regulators of socially-controlled sex change and sexual phenotype differences. Additionally, these patterns suggest TP male-typical sociosexual behaviors may depend on intermediate rather than low estrogenic signaling. We discuss these results and the possibility that an inverted-U shaped relationship between neural estrogen and male-typical behaviors is more common than presently appreciated.

KEYWORDS

aromatase, estrogen, estrogen receptor, sex change, sociosexual behavior

1 | INTRODUCTION

Sex steroid hormones play critical roles in the organization of sexual phenotypes and sex-typical behavior in all vertebrates that have been studied including fishes (Devlin & Nagahama, 2002; Guiguen et al., 2010; McCarthy & Arnold, 2011; Piferrer, 2011). One uniquely important approach to exploring the role of sex steroid hormones in sexual differentiation has taken advantage of natural variation in sexual differentiation processes, primarily sex differences. This collection of papers stems from a symposium honoring the career of

David Crews, who early on contributed to and greatly expanded this approach by seeking out "natural experiments" that could inform and expand our understanding of the links between sex steroid hormones and both sexual differentiation and the expression of sociosexual behaviors (e.g., Crews & Moore, 1986; Crews, 1991; 1993; Gutzke & Crews, 1988).

The major circulating estrogen in most vertebrates including teleost fishes is 17β-Estradiol (E_2). Although E_2 is present in both sexes, females generally maintain higher levels than males (Guiguen et al., 2010). As in mammals, testosterone (T) is also an important androgen in teleost fishes, although often primarily as a biosynthetic precursor for E_2 and 11-ketotestosterone (11KT) production (Borg, 1994; Forlano et al., 2006). Acting as an intermediate product, T is either converted to E_2 by aromatase (gonadal aromatase, Cyp19a1a, or brain aromatase, Cyp19a1b) or to 11KT by 11β-hydroxylase (Cyp11b1) and 11β–hydroxysteroid dehydrogenase 2 (HSD11b2) (Balthazart and Ball, 1998; see also Black et al., 2005). Focusing on brain aromatase, the contribution of local estrogen production and action to brain sexual differentiation is of interest in a variety of vertebrates including fishes (Balthazart and Ball, 1998; Devlin & Nagahama, 2002).

Estrogen receptors (ER) are also key contributors to sexual differentiation of gonads, secondary sexual characteristics, brain, and behavior across vertebrate animals including fishes (reviewed in Okubo et al., 2019). Although mammals have genes for two ER subtypes, three nuclear ERs have been identified in teleost fishes: a single ESR1 (hereafter ERα) and two ESR2 (hereafter ERβa and ERβb) genes (Hawkins et al., 2000). Hawkins and Thomas (2004) found this elaboration of the number of steroid receptor gene paralogs was the result of a gene duplication of ERβ in the lineage leading to teleosts. The three ERs have distinct pharmacological characteristics and different tissue distributions. The discovery of the various ER subtypes and their presence in female and male reproductive systems in similar levels led to great interest regarding their potential functions during sexual differentiation and development. In teleosts, ERs are expressed very early during embryonic development and gonadal differentiation, suggesting an important role of estrogen in sexual differentiation in teleost fishes (Guiguen et al., 1999 and Lassiter et al., 2002). ERs are also expressed in key brain regions relevant to social behavior including the preoptic area (POA), ventral tuberal hypothalamus, and telencephalic regions (Hawkins et al., 2005; Hiraki et al., 2012). These regions are hypothesized to be homologous to key areas regulating social behavior in tetrapod vertebrates (O'Connell & Hofmann, 2012).

This paper examines the relationship of estrogenic signaling to the expression of male-typical sexual and aggressive behavior in a teleost fish, the bluehead wrasse *Thalassoma bifasciatum*. This species exhibits several interesting and informative variations in sexual and reproductive patterns useful for examining neuroendocrine mechanisms subserving male-typical behaviors. These variations include socially-controlled sex change, distinct alternative male phenotypes, and timing of the display of sexual behavior driven by tidal patterns rather than time of day.

The bluehead wrasse is a small protogynous (female-to-male functionally sex changing) fish species that lives on coral reefs in the Caribbean Sea and adjacent waters. Bluehead wrasses are well studied from the perspectives of behavior and ecology (Warner & Swearer, 1991, Warner et al., 1975) as well as increasingly behavioral neuroendocrinology and neurogenomics (Lamm et al., 2015; Todd et al., 2019). Bluehead wrasses exhibit three distinct sexual phenotypes exhibiting one of two distinct color patterns. The species is named for the color pattern of the large terminal phase (TP) males that exhibit a bright blue head and green body with prominent black and white vertical bars midbody. Females and the smaller initial phase (IP) males are more drably colored, being yellow with horizontal brown stripes. Initial sexual differentiation into either a female or IP male occurs by 30 mm body standard length (SL) (Shapiro & Rasotto, 1993) and appears to be at least partially socially-controlled (Munday et al., 2006). Sex change in adult females is also socially controlled. The removal of dominant males creates a socially permissive environment that allows sex change to proceed in the largest females of a social group, as in some other protogynous species (bluehead wrasses: Warner & Swearer, 1991; other species: Fishelson, 1970; Robertson, 1972; Ross et al., 1983; Shapiro, 1980). The earliest signs of sex change in bluehead wrasses are often observed within minutes to hours as females begin to exhibit TP male-typical behavior such as aggression, inspection of the genital papilla region of IP males and females, courtship of females, and spawning with females (Warner & Swearer, 1991). This transition to dominant male-typical behavior is not dependent on gonads as even ovariectomized females can still undergo complete behavioral sex change (Godwin et al., 1996). Gonadal sex change can be rapid with ovarian atresia (breakdown of ovarian follicles) usually being advanced in three days, testicular development and permanent changes in coloration beginning in 4–6 days, and mature sperm being produced as soon as 8–10 days into the sex change process (Semsar & Godwin, 2003; Shapiro & Rasotto, 1993; Warner & Swearer, 1991, reviewed by Lamm et al., 2015; see also Todd et al., 2019).

Steroid hormones are strongly implicated in regulating natural sex change in fishes at both the gonadal and behavioral levels (Black et al., 2005, 2011; reviewed in Godwin, 2010; Lamm et al., 2015; Ortega-Recalde et al., 2020). In bluehead wrasses, a decrease in E_2 coinciding with gonadal transformation appears to be critical for behavioral transitions as E_2 implants blocked behavioral sex change under permissive social conditions following TP male removal (Marsh-Hunkin et al., 2013). Aromatase expression is high in the POA of the hypothalamus (Marsh et al., 2006), a key integrative area for male-typical sexual behavior, and is upregulated by E_2 (Marsh-Hunkin et al., 2013). Brain aromatase messenger RNA (mRNA) expression is also higher in females and declines rapidly at the onset of sex change (Todd et al., 2019; this paper).

This study builds on the findings discussed above by quantifying forebrain/midbrain expression of key genes involved in estrogenic signaling across three comparisons associated with variation in the display of dominant male-typical courtship and aggressive behavior. Specifically, we compare forebrain/midbrain mRNA abundances of

the three nuclear ER types (*ERα*, *ERβa*, and *ERβb*) and *cyp19a1b* mRNA across (i) sexual phenotypes, (ii) experimentally-induced sex changes, and (iii) tidally-driven variations in spawning behavior. Based partly on previous findings in this species, we predicted *cyp19a1b* abundances would be highest in females, intermediate in IP males, and lowest in TP males. Additionally, we predicted *cyp19a1b* mRNA abundance would decrease with female-to-male sex change and for TP males in the spawning period when sexual and aggressive behavior is increased. Given the mixed nature of findings for sex differences in ER mRNAs in both gonochoristic and sex-changing wrasse species, it was difficult to make strong predictions for *ERα*, *ERβa* and *ERβb* abundances across sexual phenotypes and sex change. We did find strong patterns in ER mRNA abundances and discuss these and the *cyp19a1b* results in the context of similar results from other well-studied species. Additionally, we discuss the possibility that an inverted-U shaped relationship between neural estrogen signaling and the display of male-typical sexual and aggressive behavior may be more common than is presently appreciated, but that technical challenges may have made uncovering this relationship challenging before recent methodological advances.

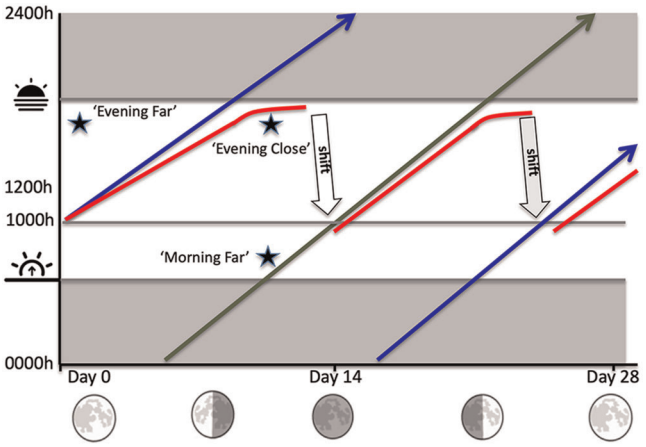

FIGURE 1 Spawning patterns and tidal cycle sampling design. Blue and green lines show clock times for the two high tides each day for the semi-diurnal tidal cycle in the Florida Keys. These change over the lunar cycle, advancing ~45 min/day (reaching 2400 h, then starting at 0000 h again on this plot). Red lines show timing of spawning for bluehead wrasses as they follow the daytime high tide. "Shift" arrows indicate a switch back to the earlier daytime high tide when this high tide occurs after approximately 1000 h. Stars indicate the timing of sampling with "Evening Far" and "Morning Far" occurring approximately 10 h from the daily spawn and "Evening Close" occurring approximately 1 h from the daily spawn

2 | METHODS

This paper presents two sets of studies where the timing of specimen recapture and sampling of brain tissues differed slightly. Study 1 focused on differences in mRNA abundances for the three ERs and *cyp19a1a* across sexual phenotypes and experimentally-induced sex change. Study 2 focused on variation with respect to the tidally-driven spawning period (Figure 1) and focused only on *cyp19a1a* mRNA abundances. These studies are described in further detail below and were approved by the North Carolina State University Animal Care and Use Committee.

2.1 | Tissue sampling

For Study 1, sex change was induced in large females in their natural habitat by removal of dominant TP males following methods used in and described for previous studies (Godwin et al., 1996, 2000; Semsar & Godwin, 2003, 2004). All bluehead wrasses over approximately 45 mm SL were captured by lift netting from small patch reefs off of Key Largo, Florida (approx. 25°20′N, 80°17′W) in May–June, 2014. TP males were immediately released back onto their reefs. Females and IP males were sexed by examination of the dimorphic genital papilla and extrusion of gametes with gentle abdominal pressure. Large females were anesthetized for approximately 6 min in 0.1 g/L MS-222 (tricaine methylsulfonate; Argent Labs) in aerated sea water, measured to the nearest 0.1 mm, and Floy-tagged (Floy Tag and Mfg., Inc.) with unique combinations of two colored beads (#11 size plastic seed beads) to allow individual recognition. Tags were inserted anteriorly on fish above the lateral line and below the dorsal fin insertion. All females were then returned to their home reefs in the afternoon on the day of capture while IP males were relocated to distant reefs. Two days later, TP males were captured before the spawning period and relocated to distant reefs to produce permissive social conditions that induce sex change in large females. Large females were allowed to undergo sex change for 1–22 days following TP male removal to achieve different gonadal stages of sex change (as defined by Nakamura et al., 1989, illustrated for bluehead wrasses in Lamm et al., 2015 and Todd et al., 2019, and described below under "Tissue Processing"). Behaviors were observed to ensure that females classified as sex changers were indeed exhibiting TP male-typical behaviors. These observations were made in 10-min focal individual increments (as described in Semsar & Godwin, 2004) and took place took place during the spawning period in which the fish were recaptured to ensure either a sex changer or female behavioral phenotype was being exhibited immediately before tissue sampling. TP male-typical behaviors include increased aggression toward conspecifics, inspection of the genital papilla area of females and IP males, courting of females, looping behavior that mimics spawning rushes, and spawning rushes with females (see Semsar & Godwin, 2004 for complete descriptions). Tagged females that showed no signs of behavioral or gonadal sex change were recaptured on the same days as sex changers to serve as controls ("control females"—these females were usually captured directly after they were observed spawning as a female). An additional and clear behavioral differentiation between sex changers and control females is space use: sex changers occupy and aggressively defend an established spawning site during the spawning period while control females instead are typically upcurrent on reefs as part of a feeding

school of IP bluehead wrasses (planktonic food arrives first at the upcurrent end of the reefs). Control females ranged from 54.4 to 66.6 mm SL ($n = 8$ overall) while sex-changing females ranged from 68.3 to 84.0 mm SL overall ($n = 19$ total across the two groups). Following recapture, fish were euthanized in an overdose of tricaine methylsulfonate (MS-222) within 2 min of capture in a waiting boat and rapidly decapitated. The brain and gonads were dissected out immediately and the brain was preserved in RNAlater (Life Technologies Inc., New York) on ice, followed by storage at −20°C, and at −80°C on return to NCSU until RNA extraction. One gonadal lobe was fixed in 4% paraformaldehyde/1X phosphate buffered saline (PBS) overnight at 4°C, followed by storage in 1X PBS until processing to assess gonadal histology and assign gonadal stages. Fifteen untagged and unmanipulated individuals were collected for this part of the study (5 females: mean length 64.9 mm, range 58.9–69.2 mm; 5 IP males: mean length 69.7 mm, range 50.6–85 mm; (5 TP males: mean length 94.3 mm, range 90.6–97.1 mm), captured between 1335 and 1645 h and their brains were processed as described above. Including these unmanipulated groups allowed a sexual phenotype comparison that did not involve handling, tagging, and the social group changes that occur in (and are inherent to) sex change experiments. Fish in the sex change comparisons were all tagged and included the control females, the early phase fish between Stages 2–3 of sex change, and the late phase fish between Stages 4–6 of sex change (these gonadal stages are described below).

2.1.1 | Study 2: Tidal cycle variation in brain aromatase mRNA sample collections

We collected mature female and TP male bluehead wrasses from the same general locations near Key Largo, FL in June 2016 under NOAA permit FKNMS-2015-051. Morning collection times ranged from 9:00 to 11:00. Evening collection times ranged from 16:00 to 20:00. The times and days of collection were chosen so that the fish would be close to or far from spawning, sampled at both morning and evening times. This created four categories: (1) "evening far", (2) "evening close", (3) "morning far", and (4) "morning close". Unfortunately, due to inclement weather we were not able to collect fish for the "morning close" category. This paper therefore focuses on the other three collection categories. A total of 54 fish were collected for this part of the study (34 females, mean 63.1 mm SL, range 47.3–73.0; 20 TP males 82.9 mm SL, range 72.0–93.5).

2.2 | Gonadal tissue processing

The fixed gonadal lobe from each sample for the control females and sex changers was processed for paraffin histology, stained with hematoxylin and eosin, and examined with light microscopy to determine the gonadal stage. Using the classifications by Nakamura et al. (1989; reviewed for bluehead wrasses in Lamm et al., 2015), control females were confirmed to have Stage 1 ovaries (normal ovary), and sex changers were undergoing Stages 2–6 of gonadal sex change (Stage 1 = normal ovary, Stage 2 = degeneration of vitellogenic oocytes, Stage 3 = degeneration of previtellogenic oocytes, Stage 4 = proliferation of Leydig cells and presumed spermatogonia, Stage 5 = onset of spermatogenesis, Stage 6 = presence of mature, tailed sperm). An additional three behavioral sex changers were still in stage 1. For Figures 3 and 4, we define "Early SC" as Stages 2 and 3 and "Late SC" as Stages 4–6 of sex change (SC).

2.3 | Brain tissue processing

For all groups, the hindbrain (including the corpus cerebelli, pons, and medulla) was removed from each brain using fine microdissection scissors under a dissecting microscope, and the entire forebrain/midbrain was processed together for quantitative reverse transcription-PCR, (qRT-PCR, see below). The forebrain/midbrain contains regions that are part of the previously mentioned social behavior network and mesolimbic reward system (O'Connell & Hofmann, 2012), which are likely to be key integrators and drivers of socially-induced sex change.

The entire forebrain/midbrain was homogenized in 1 ml TriReagent (Invitrogen) using 0.5 mm zirconium oxide beads in a Bullet Blender (Next Advance) and bromochloropropane as the phase separation reagent. A total of 325 µl of the top aqueous layer was mixed with 325 µl 70% ethanol and column-cleaned (Total RNA Purification Kit; Norgen Biotek Corp.) following the manufacturer's protocol. DNase I treatment was performed on-column for 15 min at 30°C. Total RNA was eluted in 50 µl Elution Solution. A total of 2 µl total RNA from each sample was run in an agarose gel to assess RNA integrity. Samples were assessed for concentration and purity (Nanodrop 1000; Thermo Fisher Scientific), and diluted to 250 ng/µl in RNase-free water. A total of 1 µg RNA was converted to complementary DNA (cDNA) using the SuperScript III First-Strand Synthesis System (Life Technologies, Molecular Research Center, Cincinnati, OH) following the manufacturer's protocol. A total of 4 µl total RNA was denatured for 5 min at 65°C with 1 µl dNTPs (10 mM), 1 µl oligo dT_{20} (50 µM), 1 µl random hexamers (50 ng/µl), and 3 µl RNase-free water. RNA was then converted to cDNA with the addition of 2 µl 10x RT Buffer, 4 µl $MgCl_2$ (25 mM), 2 µl DTT (0.1 M), 1 µl RNase Out (40 U/µl), and 1 µl SuperScript III RT (200 U/µl) and incubated under the following thermal conditions: 25°C for 10 min, 50°C for 50 min, and 85°C for 5 min. Samples were then incubated with RNase H at 37°C for 30 min. Samples were diluted in RNase-free water for a final concentration of 5 ng/µl and stored at −20°C. Two no-template controls containing water instead of RNA and two no-transcriptase controls containing pooled RNA and water in place of reverse transcriptase were also synthesized and diluted. Finally, two 100-µl reactions containing 18 µg pooled total RNA were synthesized to cDNA and diluted to eight concentrations from 100 to 0.01 ng/µl in 1X TE buffer for use as a standard curve.

2.4 | Quantitative reverse transcription-PCR

qRT-PCR was used to measure mRNA expression of *ERα*, *ERβa*, *ERβb*, and brain aromatase (*cyp19a1b*). The SsoAdvanced Universal SYBR Green Supermix (Bio-Rad, Laboratories, Inc.) was used for qRT-PCR with gene-specific primers. Primers for *ERα*, *ERβa*, *ERβb* were designed from the bluehead wrasse brain and gonadal transcriptome (Liu et al., 2015) using the nonconserved region of each ER to prevent multiple binding. Primers were 20 bp long and the amplicons were between 80 and 150 bp. Melting points were between 56 and 58°C. The primers were designed using Primer-BLAST (National Center for Biotechnology Information) and synthesized commercially (IDT Technologies) with the following sequences: *ERα* forward 5′-GGAGACCTTGTCCCCACAAC-3′ and reverse 5′-CCTCGGCTCT CTTCGGGATA-3′ (81 bp product), *ERβa* forward 5′-CTCCGCAGAC GATGTGGTAA-3′ and reverse 5′-GGAGAGGACGCAACTTCCAA-3′ (129 bp product), *ERβb* forward 5′-GCAGCCTCTAGGCTACAACG-3′ and reverse 5′-CGAGGGGTTCACACCATTCA-3′ (148 bp product), and *cyp19a1b* forward 5′-AGACGACAACATCGAGGGAA-3′ and reverse 5′-ACTGGGCACTGTTCTGTCAAA-3′ (136 bp product). The *EF1α* primers were forward 5′-ATCGGCGGTATTGGAACTGT-3′ and reverse 5′-CGACCATACCGGGCTTCA-3′ (67 bp product) and the *rpl9* primers were forward 5′-GACTTTGGGTTTCCGCTACA-3′ and reverse 5′-GAAGTTCCTGATCTCCACCATAC-3′ (100 bp product). Primers were tested on bluehead wrasse brain cDNA and amplicons were Sanger-sequenced (Genomic Sciences Laboratory, North Carolina State University) to confirm amplification of only the desired target transcripts.

Each primer pair produced a single melting curve peak in the presence of cDNA template and showed no amplification when water was used as a template in the reaction mix or when reverse transcriptase was omitted from the cDNA synthesis reaction (negative controls). qPCR was performed on a CFX 384 thermocycler (Bio-Rad Laboratories Inc.) in 10-μl volumes with 10 ng cDNA and 300 μM of each primer. Reaction parameters were 30 s at 95°C, 40 cycles of 95°C for 15 s, and 60°C for 30 s, and dissociation curve analysis. All reactions were performed in triplicates and reaction products for each transcript were verified by Sanger sequencing (Genomic Sciences Laboratory, NC State). For each sample, the quantity of each target gene's cDNA was obtained from a cDNA standard curve, averaged among triplicate wells, and normalized to the triplicate mean of the housekeeping gene, *EF1α* for ER mRNAs and the geometric mean of triplicate measures of *EF1α* and *rpl9* for aromatase.

2.5 | Statistical analysis

ERα, *ERβa*, *ERβb*, and *cyp19a1b* mRNA measures were compared across stable phenotypes and across sex change using one-way analysis of variance (ANOVA) and Tukey-Kramer HSD post hoc analysis. Before ANOVA analyses, we inspected the data for any clear departures from normality and confirmed homogeneity of variances using Bartlett's test. Bartlett's test indicated a nonhomogeneity of variances for *ERα* abundances across sexual phenotypes, so these data were log10 transformed and the transformed data did exhibit homogeneity of variances. We compared *cyp19a1b* mRNA abundances in females and TP males across sampling groups in the tidal study using two-way ANOVA (sexual phenotype and sampling group as factors) with orthogonal contrasts used to assess whether TP males showed an elevation in *cyp19a1b* when in closer proximity to the daily spawning period. ANOVA analyses were performed using SAS-JMP (versions 13 and 15; SAS Inc.). Statistical outliers were removed for the analysis using the ESD method (calculated using www.miniwebtool.com).

3 | RESULTS

3.1 | ER mRNA abundances across sexual phenotypes and sex change

The abundances of *ERα* and *ERβb* mRNAs varied between sexual phenotypes and over the course of sex change. Comparisons of *ERα* mRNA abundances across sexual phenotypes showed TP males having higher levels of ERα mRNA than both females and IP males (Figure 2a; ANOVA on log10 transformed data: $F_{2,12} = 5.775$, $p = 0.018$; post hoc Tukey-Kramer HSD: TP > females, IP males, $p < 0.05$; $n = 5$/group). Late SC had an approximately 50% greater relative abundance of *ERα* mRNA in the forebrain/midbrain than control females whereas Early SC did not differ from the control females in this measure (Figure 3a; ANOVA: $F_{2,19} = 6.577$, $p = 0.006$; post hoc Tukey-Kramer HSD: Late SC > control females: $p = 0.01$; Late SC = Early SC $p > 0.1$).

TP males had *ERβb* mRNA relative abundances approximately three times greater than both females and IP males (Figure 2c; ANOVA: $F_{2,12} = 11.151$, $p = 0.002$; post hoc Tukey-Kramer HSD: TP > females: $p < 0.005$; TP > IP males, $p < 0.01$). *ERβb* mRNA was also significantly higher in Late SC than control females and Early SC (Figure 3c; ANOVA: $F_{2,23} = 42.670$, $p < 0.0001$). *ERβb* mRNA abundances in Late SC reached levels approximately 2.5-fold greater than those of control, nonchanging females (post hoc Tukey-Kramer HSD: Late SC > control females, $p < 0.0001$; Late SC > Early SC, $p < 0.00001$).

Abundances of *ERβa* mRNA showed no statistically significant differences among sexual phenotypes (Figure 2b; ANOVA: $F_{2,12} = 1.386$, $p = 0.287$) nor any significant variation over the course of sex change (Figure 3b; ANOVA: $F_{2,24} = 1.767$, $p > .05$).

3.2 | Brain aromatase mRNA expression across sexual phenotypes, sex change, and tidal cycles

Study 1: Females had significantly higher mRNA abundances of brain aromatase (*cyp19a1b*) than TP and IP males (Figure 4a, $F_{2,12} = 69.656$, $p < 0.0001$; F > TP, IP: Tukey HSD $p < 0.0001$). TP males, in turn, showed higher *cyp19a1b* abundances than IP males

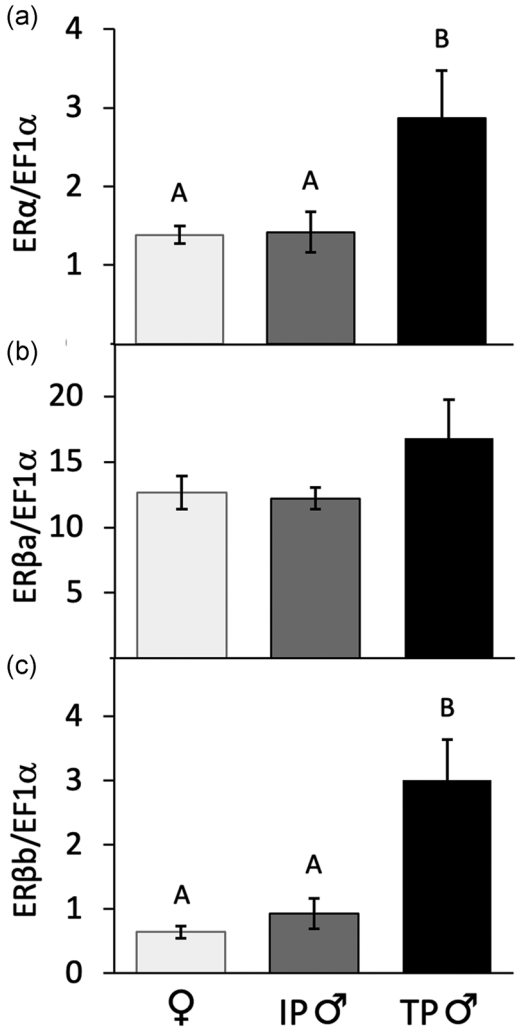

FIGURE 2 Estrogen receptor mRNA abundance variation across sexual phenotypes. Fish were untagged and unmanipulated individuals captured from study reefs. Values expressed as mean ± *SEM* ($n = 5$ per group for each transcript, different letters indicate significant differences at $p < 0.05$ as indicated by Tukey HSD post hoc tests following ANOVA). ANOVA, analysis of variance; mRNA, messenger RNA

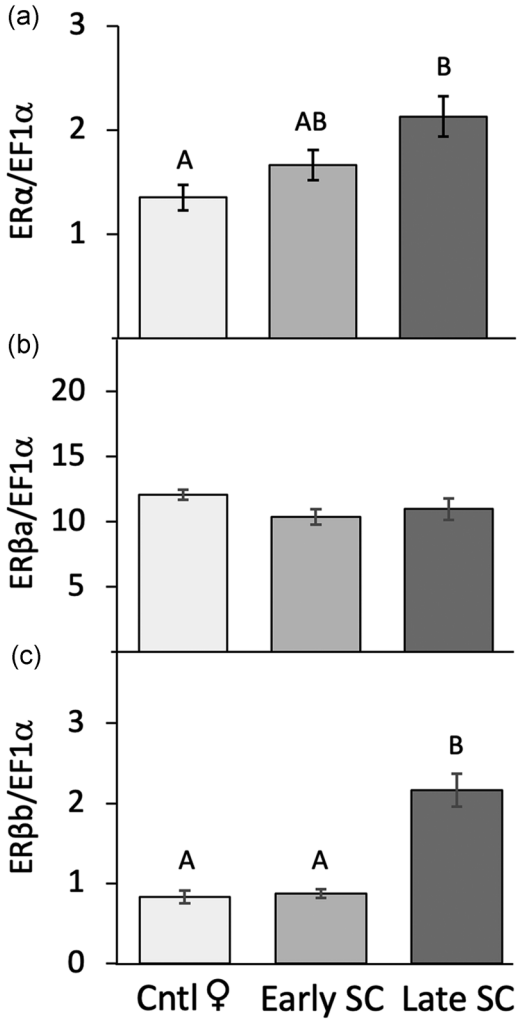

FIGURE 3 Estrogen receptor mRNA abundance variation across sex change. Values expressed as mean ± *SEM* ($n = 7$, 10, and 9 per group for ERα; $n = 8$, 10, and 9 per group for both ERβa and ERβb; different letters indicate significant differences at $p < 0.05$ as indicated by Tukey HSD post hoc tests following ANOVA). ANOVA, analysis of variance; ER, estrogen receptor; mRNA, messenger RNA

(Tukey HSD: $p < 0.005$). Brain aromatase mRNA decreased significantly with the onset of sex change, being highest in control females and then decreasing by approximately 60% in females undergoing sex change (Figure 4b). Variation was high in the early sex change group due to inclusion of three fish that were showing male-typical behavior (i.e., behavioral sex change), but not discernible ovarian histological changes yet or apparent decreases in *cyp19a1b* abundances (means similar to control females, data not shown). We excluded these three fish and then compared control females with Early SC and Late SC, which showed a highly significant decrease in *cyp19a1b* abundances with sex change (ANOVA: $F_{2,21} = 24.198$, $p < 0.0001$; Tukey-Kramer HSD: control females > [Early SC = Late SC], $p < 0.0001$, $p = 0.810$ for Early vs. Late SC).

Study 2: The abundance of *cyp19a1b* mRNA in the forebrain/midbrain differed between females and TP males and across the tidal cycle (Figure 5). Females exhibited higher abundances of *cyp19a1b* than TP males in the forebrain/midbrain across the three tidal cycle time points sampled relative to the daily spawning period (2-way ANOVA, sexual phenotype effect $F_{1,1} = 35.999$, $p < 0.0001$), consistent with previous findings (Thomas et al., 2019; Todd et al., 2019). There was also evidence of variation across sampling groups with *cyp19a1b* abundances appearing higher in the "evening close" group relative to the "evening far" and "morning far" groups (sampling group factor: $F_{2,2} = 3.134$, $p = 0.053$). There was no significant interaction between the sexual phenotype and sampling group factors ($F_{2,2} = 1.139$, $p = 0.329$). Examining only TP males across sampling group did not reveal an overall significant difference ($F_{1,17} = 5.057$, $p = 0.104$), although orthogonal contrasts of the "evening close" group relative to the "evening far" and "morning far" groups together did indicate higher *cyp19a1b* abundances closer to spawning ($p = 0.038$).

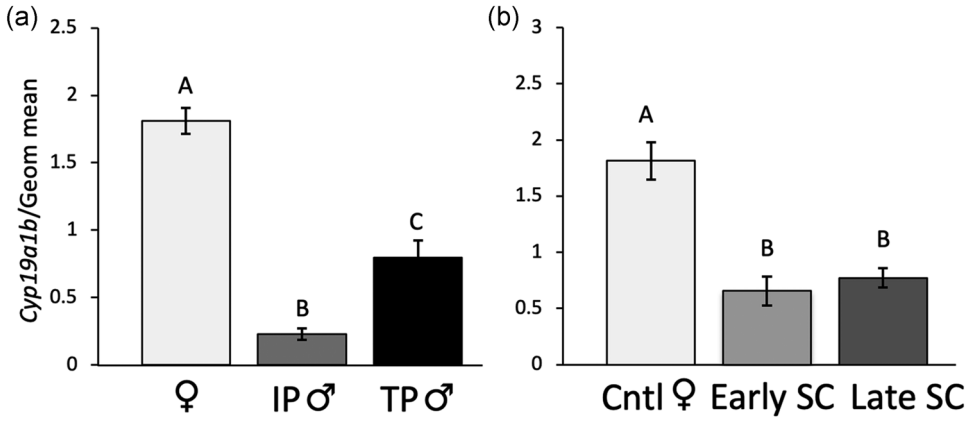

FIGURE 4 Brain aromatase mRNA (*cyp19a1b*) abundances across (A) sexual phenotypes and (B) female-to-male sex change. For sexual phenotype comparisons in panel (a), fish were untagged and unmanipulated individuals captured from study reefs. Sex changers are depicted in panel (b). Values are means ± *SEM* and different letters indicate significant differences ($p < 0.05$ by Tukey HSD; $n = 5$ for Females, IP males, and TP males in sexual phenotype comparisons; $n = 8$, 7, and 9 for Control females, Early-SC, and Late SC respectively). IP, initial phase; mRNA, messenger RNA; TP, terminal phase

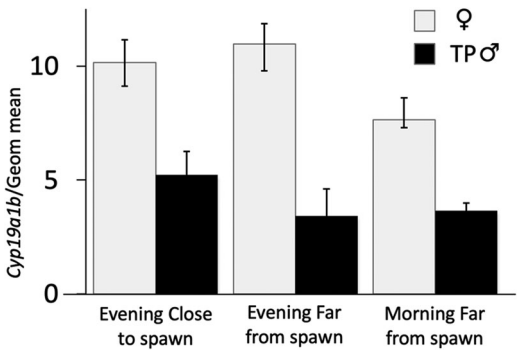

FIGURE 5 Average abundances of *cyp19a1b* for females and TP males across tidal sampling points (mean ± *SEM*). Two-way ANOVA showed a significant effect of phenotype ($F_{1,48} = 36.0$, $p < 0.001$). The second factor, sampling group, was not statistically significant ($F_{2,48} = 3.13$, $p = 0.05$), although an orthogonal contrast for TP males did reveal higher abundances for the "Evening Close" relative to the "Evening Far" and "Morning Far" categories ($p < 0.05$) ($n = 16$, 6, and 12 for females and 5, 4, and 10 for TP males in the Evening Close, Evening Far, and Morning Far groups, respectively)
Appendices: N/A. TP, terminal phase

4 | DISCUSSION

Estrogenic signaling is an important regulator of sexual differentiation and sexual behavior across vertebrate animals including fishes, but there also appear to be important differences in the nature and mechanisms of estrogen action across taxa (reviewed in McCarthy & Arnold, 2011; Okubo et al., 2019; Ortega-Recalde et al., 2020). The findings presented in this paper are consistent with the recognized important role for estrogen signaling regulating sociosexual behavior in teleosts. However, the several comparisons made possible by sexual variation in the bluehead wrasse system suggest a complex and possibly nonmonotonic relationship between neural estrogen signaling and sexual and aggressive behavior typical of the large and territorial TP males. These variations include experimentally-induced adult functional sex change, between discrete within-sex alternative mating phenotypes, and daily variation in reproductive behavior that follows tidal rather than time-of-day cycles. Below, we first discuss our findings regarding estrogen signaling in relation to behavioral variation in bluehead wrasses and some other teleosts. We then briefly consider whether a nonmonotonic, potentially "Inverted-U" shaped relationship between male-typical sociosexual behaviors and estrogen signaling could be more common across species than currently appreciated and whether more recent technical advances in our ability to study estrogen signaling can help address this question.

4.1 | ER mRNA abundances across sexual phenotypes and sex change

The abundances of *ERα* and *ERβb* mRNA changed in the bluehead wrasse forebrain/midbrain both across sexual phenotypes and over the course of experimentally-induced female-to-TP-male sex change (Figures 2 and 3). Several studies have addressed ER expression in the brain of teleosts (Atlantic croaker, Hawkins et al., 2005; zebrafish, Menuet et al., 2004), and sex differences have also been described (medaka, Hiraki et al., 2012; *Halichoeres trimaculatus*, Kim et al., 2002), but no study to date has examined all three ER mRNAs over the course of sex change with a focused qPCR approach. Evidence from other teleosts indicates gonadal status can strongly affect ER expression. For example, the overall expression levels of ER mRNAs in the medaka brain were correlated with phenotypic sex and not with genetic sex, being instead very strongly responsive to the steroid hormone environment and indicating that the sex differences were not attributable to effects of sex chromosomes (Hiraki et al., 2012; see also Okubo et al., 2019). While we do not have measures of circulating E_2 for bluehead wrasses, females do have approximately four- to

fivefold higher plasma E_2 levels than TP males in the congeneric *Thalassoma duperrey* (Nakamura et al., 1989). Therefore, it appears likely that circulating estradiol levels and neural expression of ER mRNAs are overall negatively correlated in females and TP male bluehead wrasses: low E_2 in TPs and higher expression of ER mRNAs; high E_2 in females and lower expression of ER mRNAs (although not for *ERβa* mRNA).

ER mRNA expression has been investigated in other species across changes in social status. The well-studied African cichlid fish, *Astatotilapia burtoni*, exhibits a pronounced social regulation of reproductive function, and dominant males expressed higher levels of *ERβa* and *ERβb* than subordinate males (Burmeister et al., 2007), suggesting *ERβa* and *ERβb* are regulated by social status and reproductive maturity and could be upregulated due to low circulating estradiol levels. In contrast to these results, a more recent study in *A. burtoni* found little difference between *ERβa* and *ERβb* mRNAs in various microdissected brain regions between subordinate and dominant males, while levels of *ERα* mRNA did show differences (Maruska et al., 2013). Ascending male *A. burtoni* showed increases in both circulating E_2 and in ERα mRNA abundances for several brain areas (although not the preoptic area). The authors suggest increased estrogen sensitivity in specific socially-relevant nuclei may be important for the expression of behaviors (territorial and/or reproductive) and physiological changes that occur during transition to social dominance (Huffman et al., 2012; O'Connell and Hofmann, 2011). Interestingly, in our study the increase in relative abundances of *ERα* and *ERβb* mRNAs observed over sex change did not appear until the later stages (4–6) of sex change (Figure 3). The lack of increase in the early stages (1–3) could be due to a delayed response to the decrease in neural synthesis of estrogen by aromatase. Changes in patterns of regulatory control could also be occurring. Hiraki et al. (2012) found that steroid receptor (ER and AR) mRNA levels were positively correlated with circulating steroid levels in male medaka, but negatively correlated in female medaka.

4.2 | Estrogenic regulation of sociosexual behaviors

While estrogens play a key stimulatory role in producing male-typical neuronal and behavioral phenotypes in many mammals and birds (reviewed in Balthazart et al., 2009; McCarthy & Arnold, 2011; Trainor et al., 2006), the opposite has been suggested for at least some fishes. One example is the bluebanded goby *Lythrypnus dalli* where brain aromatase activity declines rapidly at the onset of female-to-male sex change (Black et al., 2005, 2011). By contrast, brain aromatase mRNA increases over the course of male-to-female sex change in the anemonefish *Amphiprion bicinctus* (Casas et al., 2016). The plainfin midshipman (*Porichthys notatus*) does not show sex change, but does exhibit discrete alternative male phenotypes. Hindbrain aromatase activity is higher in females and nonterritorial males of this species than in large territorial males (Forlano et al., 2006; Schlinger et al., 1999). Studies of sex-changing species also indicate E_2 administration inhibits female-to-male sex change and/or male-typical behaviors (reviewed by Godwin, 2010; Lamm et al., 2015; Ortega-Recalde et al., 2020). Reductions in estrogen signaling also appear important for sex change in bluehead wrasses as gonadal aromatase mRNA (*cyp19a1a*) decreases dramatically as the ovary becomes a testis (Thomas et al., 2019; Todd et al., 2019), E_2 implants block behavioral sex change (Marsh-Hunkin et al., 2013), and forebrain/midbrain aromatase mRNA levels decrease significantly during the early stages (2–3) of gonadal sex change (Figure 4). However, several observations presented here suggest the relationship between estrogenic signaling and TP male typical sexual and aggressive behavior in the bluehead wrasse may be complex, nonmonotonic, and not consistent with a strict inhibition model. Below, we discuss these observations and how they may be similar to the relationship between male-typical behavior and estrogenic signaling described in Japanese quail (Ubuka & Haraguchi, Tobari, et al., 2014) and possibly also some other well-studied species.

Patterns of forebrain/midbrain *cyp19a1b* and ER mRNA abundances do not appear consistent with a model of strict inhibition of TP male-typical behavior by estrogenic signaling in bluehead wrasses for several reasons. *First*, declines in *cyp19a1b* were not apparent in the earliest stages of sex change when behavioral change had begun but gonadal change had not (Stage 1, see also Todd et al., 2019 where a RNA sequencing approach was used), although caution is warranted on this point due to small sample sizes. *Second*, while *cyp19a1b* mRNA abundances were significantly higher in females than TP males and control females relative to sex changers (Figure 4) (as also documented previously using RNA sequencing in Todd et al., 2019), TP males had higher *cyp19a1b* levels than IP males that are nonaggressive and do not display courtship behavior. Additionally, we found some evidence that *cyp19a1b* levels increase rather than decrease as TP males approach the spawning period when courtship and aggression is exhibited (Figure 5). Last, we found an increase in forebrain/midbrain ERα and ERβb mRNA abundances over the course of sex change in the bluehead wrasse, suggesting a potential increase in sensitivity to E_2 (Figure 3).

Would a nonmonotonic and potentially "Inverted-U" shaped relationship between neural estrogen signaling and male-typical sociosexual behaviors be surprising? Such effects have been documented most notably with glucocorticoids and learning and memory processes with the well-known inverted-U shaped Yerkes-Dodson curve (Lupien & Lepage, 2001). Nonmonotonic patterns are also known for sex steroid modulation of sociosexual behavior including with respect to neural estrogen signaling. A full review of such nonmonotonic effects is beyond the scope of this paper, but a few examples are noteworthy. Borland et al. (2019) propose an "inverted U hypothesis" to explain variations in regulation of social reward processes by oxytocin within and between sexes in both rodent models and humans. For steroid hormones, McFadden (2011) summarizes several examples of nonmonotonic relationships between testosterone exposure and masculinization of the phenotype in rodents and ferrets. Of most direct relevance here, Ubuka et al. (2014) demonstrated that male Japanese quail show a robust daily rhythm in aromatase activity and tissue E_2 levels in the hypothalamus and that increased neuroestrogen inhibits male-typical sociosexual

behavior. These sociosexual behaviors are displayed robustly in the morning when measured tissue E_2 levels in hypothalamus are low, but decrease steadily through the day as these E_2 levels increase. These circadian-related behavioral differences were recapitulated by centrally administering gonadotropin-inhibitory hormone (GnIH, shown in this study to increase aromatization and hypothalamic E_2 levels) or injecting E_2 intracerebroventricularly. Conversely, Ubuka and colleagues note that the requirement for a minimum level of neural E_2 for the display of male-typical sociosexual behaviors and overall inverted-U shaped relationship in quail is suggested by other studies showing that (i) systemically-administered E_2 can induce these behaviors in castrated or reproductively inactivated birds (Adkins et al., 1980) and (ii) acute treatment of reproductively-active male quail with the aromatase inhibitor vorozole both blocked aromatase activity in the preoptic area of the hypothalamus and inhibited sexual behavior (Cornil et al., 2006).

The measurement of E_2 levels in hypothalamic tissue (rather than blood) and administering E_2 directly to the brain via i.c.v. injection were two strong methodological aspects of the study by Ubuka and colleagues discussed above. These approaches are important because it is becoming increasingly apparent that systemic levels of E_2 often do not accurately reflect brain levels, which can be much higher (Hojo et al., 2011; Jalabert et al., 2021; Taves et al., 2011) as well as rapidly regulated in a brain region specific manner (de Bournonville et al., 2020; Liere et al., 2019; see Cornil & de Bournonville, 2018 and Balthazart, 2019 for reviews). Additionally, systemic injections of E_2 may not effectively and predictably alter neural levels of this hormone (Liere et al., 2019). Apart from the avian-focused examples noted above, tissue E_2 measures and i.c.v. administration have not yet been widely applied to studying the relationship of estrogenic signaling and male sociosexual behavior to our knowledge. Therefore, the prevalence of nonmonotonic, inverted U-shaped relationships as documented in Japanese quail and hypothesized here for bluehead wrasses has not been broadly assessed including apparently for widely-used rodent models, but this would be a useful direction for future studies.

4.3 | Summary and future directions

This study documents significant differences in abundances of *ERα*, *ERβb* and brain aromatase mRNA in the forebrain/midbrain level both across sexual phenotypes and with sex change, while differences were not found for *ERβa*. The patterns described do not appear to fit a model of strict inhibition of TP-male courtship and territorial behavior and may instead reflect a nonmonotonic relationship between neural estrogen signaling and TP male-typical sociosexual behavior. While future studies are needed to better characterize these patterns, the results do add support for neural estrogen signaling likely being a key regulator of socially-controlled sex change and sociosexual behavior more generally in bluehead wrasses. Important next steps include use of microdissection to provide greater neuroanatomical resolution to measures of ER and brain aromatase mRNA abundances and measures of E_2 levels in brain tissue.

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CONFLICT OF INTERESTS

The authors declare that there are no conflict of interests.

DATA AVAILABILITY STATEMENT

The data that support the findings of this study are available from the corresponding author upon reasonable request.

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