ABSTRACT

PRIM, JULIANNA H. Estrogen Receptor Expression in the Brain of the Sex-changing Bluehead Wrasse, *Thalassoma bifasciatum*. (Under the direction of John Godwin).

This study examined the expression of estrogen receptors in the brain of the bluehead wrasse (*Thalassoma bifasciatum*), a teleost fish that exhibits socially-controlled behavioral and gonadal sex change. The goal is to better understand the role of estrogen receptor signaling in the sex change process and explore the potential role of estrogen receptor subtypes (ERα, ERβa, and ERβb) in mediating the behavioral effects of estrogens. Estrogen signaling has recently emerged as a likely key regulator of sex change at both the gonadal and brain levels. However, a comprehensive study investigating the expression of all three ER subtypes during sex change in association with histological change has been lacking for hermaphroditic fishes. We analyzed the sequence of the teleost estrogen receptors (ERα, ERβa, and ERβb) and used quantitative reverse-transcriptase PCR (qPCR) of brain cDNA to compare ER mRNA relative abundance between sexes and across sex change. Our results found significant differences in ERβb relative abundances among sexual phenotypes (female, IP (initial phase) male, TP (terminal phase) male), with TP males having higher levels and an increase over the course of sex change with late sex changers having significantly higher levels. ERα showed no significant difference among sexual phenotypes, but over the course of sex change, the late stage fish had higher ERα relative abundances than control females. Meanwhile, relative ERβa abundances showed no significant differences among sexual phenotypes or over sex change. Our results suggest alterations in estrogen signaling in the brain are likely to be key regulators of the sex change process.

Estrogen Receptor Expression in the Brain of the Sex-changing Bluehead Wrasse, *Thalassoma bifasciatum*

by
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DEDICATION

This thesis work is dedicated to the memory of my grandfather, Gorrell Clinton Prim, who will forever be my hero. I will always remember his unconditional love and constant encouragement. He believed in me when I didn't even believe in myself. Every day I am grateful for his positive influence and presence in my life.

BIOGRAPHY

Julianna Prim was born on December 3, 1990. She graduated from Starmount High School in 2009 as Valedictorian and four time state champion in swimming. She completed her undergrad degree in Biology at North Carolina State University and continued to pursue her Master's in Zoology under the direction of John Godwin. While in college, Julianna swam on the varsity team for four years and played on the women's basketball team one year achieving All-ACC and Academic All American Honors as well as top ten scholar athlete. She was also named MVP and broke numerous school records in swimming. Julianna received the ACC Top 6 for service awards for her community service in mentoring programs and outreach events. She is a member of the Phi Kappa Phi and Phi Beta Kappa honor fraternities.

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CHAPTER 1

Introduction

The sex steroid hormones including estrogens and androgens, have important roles in the sexual differentiation (the process of gonad development after sex has been determined) and organization of sex-typical behavior in all vertebrates that have been studied. Estrogens and androgens regulate ovarian and testicular differentiation respectively (Devlin and Nagahama 2002; Guiguen et al. 2010; Piferrer 2011). The major estrogen in teleost fish is 17β-Estradiol (E_2) and the major male-specific androgen is 11-Ketotestosterone (11KT) (Devlin and Nagahama 2002). Although present in both sexes, females generally maintain higher E_2 levels than males (Hiraki, 2011). While the major mammalian androgen, testosterone (T) is found in teleost fish, its primary role is thought to be as a precursor for E_2 and 11KT production. Acting as an intermediate product, T is either converted to by E_2 by aromatase (gonadal, Cyp19a1a, or brain, Cyp19a1b) or to 11KT by 11β–hydroxysteroid dehydrogenase 2 (HSD11b2) (Balthazart et al, 1998).

Local production of steroid hormones in the brain is of increasing interest with respect to control of numerous behavioral and physiological processes from male sexual behavior to brain sex differentiation in a variety of species (Balthazart et al, 1998). For example, in rainbow trout and tilapia, aromatase is repressed during male differentiation (Devlin and Nagahama 2002). Along with the long term effects E_2 and 11KT have on the teleost brain and behavior (days to week), they can also produce rapid (seconds to minutes) behavioral changes (Remage-Healey and Bass 2007). The widespread and abundant expression of hsd11b2 and cyp19a1b in both the male and female adult teleost brain (100-

1000 times greater than in mammals or birds) suggest conversions to E_2 and 11KT are important in regulating teleost neural functions and plasticity (Marsh et al. 2006; Alderman and Vijayan 2012). Even the male brains have high amounts of estrogen (Hiraki et al, 2011).

Neurosteroidogenesis also appears to be important in regulating sex change. In the protogynous blue-banded goby (*Lythypnus dalli*), brain aromatase activity decreased in sex changing females rapidly (hours) after removal of males, which had initiated the female-to-male sex change (Black et al. 2005). This decrease in whole brain aromatase activity is preceded by behavioral changes like increased aggression during sex change (Black et al., 2011). Estradiol can block gonadal sex change in some fishes and the lack of E_2 will stimulate it in others (Guiguen et al. 2010). For example, plasma estrogen levels decrease very early into the sex change process in the congeneric saddleback wrasse (Thalassoma bifasciatum; Nakamura et al., 1989). In bluehead wrasses, a decrease in E_2 appears to be necessary for the sex change process to begin. When females were given E_2 implants under conditions permissive for sex change (i.e. TP males removed), behavioral sex change did not occur (Marsh-Hunkin et al., 2013). In addition, treatment with E_2 increased aromatase mRNA in the preoptic area of the hypothalamus and the female POA also had significantly higher relative abundances of aromatase mRNA than TP males. (Marsh-Hunkin et al. 2013).

Estrogens exert their effects largely via cytosolic estrogen receptors (ERs) present in target tissues (Coumailleau et al, 2015). Estrogen receptors are members of the nuclear receptor superfamily of ligand-activated transcription factors. All members of this family have a similar structure with six distinct domains: the activation domain, the highly conserved DNA-binding domain, variable hinge region, the well conserved ligand-binding domain, and the variable C-terminus (Krust et al., 1986; Hewitt et al, 2002; Hawkins et al., 2004).

Although mammals have one gene for two estrogen receptor subtypes (ESR1 and ESR2), in teleost fishes three estrogen receptors of the regulatory transcription factor type have been identified- a single ESR1 (ERα) and two ESR2 (ERβa and ERβb) genes (Hawkins et al., 2000). Hawkins et al (2004) found this elaboration of the number of steroid receptor gene paralogs was the result of a gene duplication of ER® in the lineage leading to teleosts (Hawkins et al, 2004). The three ERs have distinct pharmacological characteristics and different tissue distributions. The discovery of the various ER subtypes and their presence in the female and male reproductive systems in similar levels has produced 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 fish (Guiguen et al., 1999 and Lassiter et al., 2002).

Estrogen receptors are expressed in key brain regions relevant to social behavior including the preoptic area, ventral tuberal hypothalamus, and telencephalic regions (Hawkins et al., 2005; Hiraki et al., 2012). These regions are thought to be homologous to key areas regulating social behavior in vertebrates (O'Connell and Hoffman, 2012). For example, the social behavior network was first described in mammals but has expanded in other vertebrate classes (Munchrath et al, 2010). The brain regions in the network express steroid hormone receptors and include the preoptic area, anterior hypothalamus, ventromedial hypothalamus, medial amygdala, bed nucleus of the stria terminalis, periaqueductal gray, and the lateral septum (Munchrath et al, 2010). Every vertebrate class studied shows steroid receptor expression in these areas (Munchrath et al, 2010).

In our study, we investigated estrogen receptor expression throughout sex change in the bluehead wrasse. The bluehead wrasse is a small protogynous fish species that live on coral reefs in the Caribbean Sea and adjacent waters. There are three different sexual phenotypes with two colorations. The fish gets its name from the large TP (terminal color phase) males: a bright blue head is separated from a green body by three vertical bars, two of which are black and the other white. All female and the smaller IP (initial phase) males have a distinctly different coloration: yellow and brown striped. Initial sexual differentiation into either a female or initial-phase (IP) male occurs at approximately 30 mm standard length (Shapiro and Rasotto 1993) and is at least partially socially-controlled (Munday et al. 2006). For instance, on a large reef up to fifty percent of juveniles may develop into IP males whereas on a small reef almost all juveniles develop into females (Warner 1984). TP males develop later in life through a sex or role changes in large females and IP males, which involve dramatic alterations in behavior and morphology (Semsar et al. 2001).

Bluehead wrasses spawn at approximately midday year around, usually near high tide, by releasing gametes into water column (Feddern 1965). Females usually spawn once every 1-3 days (Feddern 1965), while males can spawn numerous times per day (Warner et al. 1975). The female-mimic IP males spawn on a daily basis with groups of other IP males or sneak on pair spawns between the TP males and females. Usually the group spawning occurs on large reefs with 5-20 IP males, while on small reefs the IP males streak into a pair spawning with a TP male and female. The IP male behavior closely resembles females in that they show little aggression and courting. Territorial TP males on the other hand will vigorously chase all conspecifics away from the mating site, except females arriving to spawn who they court by swimming in tight circles while rapidly vibrating their pectoral fins (Warner and Swearer 1991). There are also non-territorial TP (NT-TP) males who have TP coloration but do not defend a spawning site, rarely courting females or chasing conspecifics. They rapidly occupy a territory when one becomes available and will then partake in male typical behavior (Semsar and Godwin, 2004).

The bluehead wrasse exhibits social control of sex change, in that the absence of T-TP and NT-TP males can initiate sex change (Warner and Swearer 1991). Acting as a "trigger" in bluehead wrasse, along with other protogynous species, e.g., lyretail anthias (*Anthias squamipinnis*; Fishelson 1970; Shapiro 1980) and bluestreak cleaner wrasse (*Labroides dimidiatus*; Robertson 1972), this removal of dominant males creates a permissive social environment for sex change in the largest, highest-ranking females of a social group (Robertson 1972; Ross et al. 1983; Warner and Swearer 1991). The earliest signs of sex change are visible very rapidly (within minutes to hours) as females begin to exhibit male typical behavior such as aggression, inspection of the genital papilla region of IP males and females, courtship of females, and spawning with females (for a complete description of TP male-typical behaviors, see Semsar and Godwin 2004). This display of male typical behavior is socially controlled (removal of TPs) and not dependent on gonads as even ovariectomized fish still undergo complete behavioral sex change (Godwin at al, 1996). Gonadal sex change is rapid: ovarian atresia is advanced by three days, testicular development begins by four to six days and permanent change in coloration begins to occur, mature sperm are produced by day eight to ten of sex change (Warner and Swearer 1991; Shapiro and Rasotto 1993; Semsar and Godwin 2003).

Social control of sex change in reef fish was first documented over forty years ago (Warner and Swearer 1991) and since that time many experiments have been done investigating neural pathways affected by environmental changes. Gonadal sex change has been investigated in several protogynous species as an example of sexual lability and phenotypic plasticity with the radical morphological changes and the restructuring of tissues from a functional ovary to a functional testis (reviewed by Godwin 2010; see also Muncaster et al. 2013; Nozu et al. 2013). The bluehead wrasse has become a valuable model in studying these behavioral and physiological changes for numerous reasons. As previously mentioned, females undergo a rapid sex change at the removal of dominant males with behavioral changes in minutes and fully functional testes by day 8-10 (Warner and Swearer 1991). The daily spawning all year allows for flexibility in sex change experiments. Bluehead wrasse can be caught relatively easily by lift netting, facilitating manipulative sex-change experiments and hormonal manipulations on the fish's home reefs in nature (Feddern, 1965; Warner and Swearer, 1991; Godwin et al. 1996; Semsar et al. 2001; Semsar and Godwin 2003 & 2004; Marsh-Hunkin et al. 2013). If the home reef is sufficiently isolated, bluehead wrasses will not leave these patch reefs (Warner 1984), allowing each patch reef to serve as a distinct 'experimental enclosure' within the fish's natural environment. Marking methods using unique color combinations of beaded Floy tags make it possible to track fish on their home reef and record behaviors (e.g., Godwin et al., 1996; Semsar and Godwin, 2003; Marsh-Hunkin et al., 2013).

While steroid hormones are strongly implicated in regulating natural sex change in fishes, the exact mechanisms involved are still unclear. This study examines estrogen receptor mRNA expression in the brain of the different sexual phenotypes of bluehead wrasses and changes that occur with socially-induced sex change using qPCR analysis. A better characterization of estrogen signaling through these receptors may enhance our understanding of the molecular mechanisms that mediate sex change in this important model species for sex change.

Methods

Tissue Sampling

Sex change was first induced in large females by removal of dominant TP males following methods used in previous studies (Godwin *et al.*, 1996; Godwin *et al.*, 2000; Semsar and Godwin, 2003 & 2004). All bluehead wrasses over approximately 45 mm standard length (SL) on a given reef were first captured via lift net and transported back to the waiting boat (approx. 25o20'N, 80o17'W). Through examination of the dimorphic genital papilla and extrusion of gametes with gentle abdominal pressure, females and IP males were sexed. Large females were anesthetized for approximately 6 min in 0.1 g/L MS-222 in sea water, measured to the nearest 0.1 mm, and Floy-tagged (Floy Tag and Mfg., Inc., approx. 25o20'N, 80o17'W) with unique combinations of two colored beads (#11 size plastic seed beads). 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 prior to the spawning period and relocated to distant reefs to 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 in Nakamura et al, 1989). Behaviors were observed prior to recapture to ensure these females were exhibiting TP male-typical behaviors. Male-typical behaviors include increased aggression toward conspecifics, inspection of the genital papilla of females and IP males, courting of females, looping behavior that mimicked spawning rushes, and spawning rushes with females (see Semsar and Godwin, 2004 for complete descriptions). Tagged females that showed no signs of behavioral or gonadal sex change (usually directly after spawning as female) were captured on the same days as sex changers to serve as controls ('control females'). Fish were euthanized in an overdose of *MS-222* within 2 min of capture and rapidly decapitated. The brain and gonads were dissected immediately in a waiting boat. The brain was preserved in RNAlater (Life Technologies, Inc. NY) on ice, followed by storage at −20 °C and eventually −80 °C until RNA extraction. One gonadal lobe was fixed in 4% paraformaldehyde/1X PBS overnight at 4 °C, followed by storage in 1X PBS until histological processing. Five untagged females (mean length 64.9 mm, range 58.9-69.2 mm), IP males (mean length 69.7 mm, range 50.6-85 mm), and TP males (mean length 94.3 mm, range 90.6-97.1 mm) were also captured (6/12/14-6/16/14 between 13:35-16:45), and their brains were processed as above. Including the untagged group allowed us to control for handling, tagging, and the stresses that come with it. In our sex changing comparisons the control fish are tagged females, the early phase fish are between stages 1-3 of sex change, and the late phase fish are between stages 4-6 of sex change.

Tissue processing

The hindbrain (including the corpus cerebelli, pons, and medulla) was removed from each brain, and the entire forebrain/midbrain was processed for qRT-PCR. The forebrain/midbrain contains regions that are part of the previously mentioned social behavior network and mesolimbic reward system (O'Connell and Hofmann, 2011), which are likely to be key integrators and drivers of socially-induced sex change. The fixed gonadal lobe from each sample for the control females and sex changers was processed for paraffin histology and stained with hematoxylin and eosin (Histology Laboratory, College of Veterinary Medicine, NCSU) to determine the gonadal stage. Using the classifications by Nakamura *et al.* (1989), 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).

The entire forebrain/midbrain was homogenized in 1 mL TriReagent (Invitrogen) using bromochloropropane as the phase separation reagent. 325 µL of the top aqueous layer was mixed with 325 µL 70% ethanol and column-cleaned (Total RNA Purification Kit, Norgen Biotek Corp., Ontario, Canada) following the manufacturer's protocol. DNase I treatment was performed on-column for 15 min at 30º C. Total RNA was eluted in 50 µL Elution Solution. Two 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. One µg total RNA was converted to cDNA using the SuperScript III First-Strand Synthesis System (Life Technologies, Molecular Research Center) following the manufacturer's protocol. Four µL total RNA was denatured for 5 min at 65º 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: 25º C for 10 min, 50º C for 50 min, and 85º C for 5 min. Samples were then incubated with RNase H at 37º C for 30 min. Samples were diluted to 200 µL in RNase-free water for a final concentration of 5 ng/µL and stored at -20º C. Two no-amplicon controls (NAC) containing water instead of RNA and two no-transcriptase controls (NTC) containing pooled RNA and water in place of reverse transcriptase were also synthesized and diluted to 200 ng/µL. Finally, two 100-µL reactions containing 18 µg pooled total RNA were synthesized to cDNA and diluted to eight concentrations from 100 – 0.01 ng/µL in 1X TE buffer for use as a standard curve.

Quantitative Reverse Transcription- PCR (qRT-PCR)

Quantitative RT-PCR was used to measure mRNA expression of ERα, ERβa, and ERβb from whole brain cDNA. The SsoAdvanced Universal SYBR Green supermix (Bio-Rad) was used for qRT-PCR with gene-specific primers. Primers for ERα, ERβa, and ERβb were designed from the bluehead wrasse brain and gonadal transcriptome (Liu, et al unpublished) using the nonconserved region of each ER to prevent multiple binding. The current ER nucleotide sequences on NCBI were incomplete so we used the transcriptome to design primers. Primers were 20 bp long and the amplicons were between 80-150 bp. Melting points were between 56-58 C. The primers were designed using primer blast (IDT Technologies, Coralville, IA) and synthesized commercially with the following sequences: Erα forward 5′-GGA GAC CTT GTC CCC ACA AC-3′ and reverse 5′- CCT CGG CTC TCT TCG GGA TA′ (81 bp product), Erβa forward 5′- CTC CGC AGA CGA TGT GGT AA-3′ and reverse 5′- GGA GAG GAC GCA ACT TCC AA-3′ (129 bp product), and Erβb forward 5'- GCA GCC TCT AGG CTA CAA CG -3' and reverse 5'- CGA GGG GTT CAC ACC ATT CA- 3' (148 bp product). The EF1α primers were forward 5'- ATC GGC GGT ATT GGA ACT GT -3'and reverse 5'- CGA CCA TAC CGG GCT TCA- 3' (67 bp primers). (Table 1.1)

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). PCR was performed on a CFX 384 thermocycler (Bio-Rad, Raleigh, NC), and the reaction progress in 10-μl volumes was monitored by fluorescence detection at 450–690 nm during each annealing step. Reaction parameters were 30 seconds at 95 C followed by 40 cycles of 95 C for 15 seconds and 60 C for 30 seconds and followed by a dissociation curve analysis. All reactions were performed in triplicates and several reaction products for each ER were verified by DNA sequencing (GSL, NC State). By using the kinetics of individual reactions, estimates of efficiency and CT are independent of the specific equipment used to perform PCR, and data can be more reliably compared across plates. The relative amount of a target gene mRNA was then normalized to the mean of the housekeeping gene EF1α that was also measured in the sample.

Statistical Analysis

Using the SAS-JMP version 11 (SAS, Cary, NC), One-way ANOVAs were applied to ERα, βa, and βb to compare sex differences and variations over sex change respectively. The first one-way ANOVA compared means of untagged females, IP males, and TP males. The second one-way ANOVA compared means of control females and two broad phases of sex change: early (stage 1-3), and late (stage 4-6). Following detection of a significant difference, means were compared using Tukey Kramer HSD. All outliers were removed for the analysis (ESD method, calculator miniwebtool).

Results

We compared the inferred nucleotide sequences for ERα, ERβa, and ERβb in bluehead wrasses were compared to ER sequences in four other fish species and humans (Table 1.2). Inputting our transcriptome sequence into NCBI blastx, the nucleotide sequences exhibited similar sequence identity with the other species for the corresponding ER (ERα, ERβa, and ERβb). Our transcriptome includes 4012 nucleotides for ERα, 2117 for ERβa, and 2611 for ERβb. It should be noted that the full sequence of the ERβa has not been identified (Liu, et al unpublished). First, we did sequence alignment of the full deduced amino acid sequences for these three cDNAs confirmed their homologies to the ERα (EsR1), ERβa (Esr2a or Esr3), and ERβb (Esr2b) receptor cDNAs identified previously from yellow Croaker (ERα 84%,ERβa 62% , and ERβb 84%), Chinese Wrasse (94%, 63%, 91%), Zebrafish (73%, 67%,62%), and Atlantic Croaker (84%, 73%,78%). We then compared specifically the DNA-binding domain (DBD) and the Ligand-binding domain (LBD) amino acid sequences between the bluehead wrasse and each of the five other species (Table 1.2). While the ligand binding domain had 60% identity with the other species, the DNA binding domain has over 90% sequence identity showing how highly conserved this domain is in the ERs.

The entire lengths of the sequences for ERα, ERβa, and ERβb were used for alignment with Clustal Omega (website, EMBL-EBI). Sequences were aligned by using an alignment technique that avoids the arbitrary choice of a single gap-change cost by aligning at several values within a defined window. Aligning the amino acid sequences of the ERα, ERβa, and ERβb of the bluehead wrasse and other species allowed us to define domains using markers. For instance the DNA-binding domain of ERs is composed of two C4-type zinc fingers containing a group of four Cys residues which coordinates a single zinc atom (Menuet et al, 2004). Estrogen receptors interact with specific DNA sites upstream of the target gene and modulate the rate of transcriptional initiation. Estrogen binding to the receptor triggers the dimerization and the subsequent binding of the receptor dimer to an estrogen response element, which is a palindromic inverted repeat: 5'GGTCA…TGACC-3', of target genes. (Socorro et al, 2000) (Fig. 1.1).

We found a significant difference in abundance of ERα and ERβb between the sexes and over the course of sex change. Late sex changers have a fifty percent greater measure of relative abundance of ERα mRNA in the forebrain than control females whereas early sex changers_do not differ from the females or TP males in this measure (Fig. 1.2a; ANOVA $F_{2,19}$= 5.830, p = 0.0101; posthoc Tukey-Kramer HSD: TP > females: p < 0.01; TP = IP males p=.1089, 0.10>p>0.05). Also in the ERα female, IP male, and TP male comparisons, a trend was observed where TP males had the higher levels of ERα than females but this was not statistically significant (fig. 1.3a; ANOVA $F_{2,19}$= 5.830, p = 0.0521; post hoc Tukey-Kramer HSD: TP= females: p 0.10> 0.>; TP = IP males, 0.10>p>0.05).

TP males have ERβb mRNA relative abundances approximately three times greater than either females and IP males (fig. 1.3c; ANOVA $F_{2,19}$= 5.830, p = 0.0027; posthoc Tukey-Kramer HSD: TP > females: p < 0.05; TP > IP males, p=0.0069, p<0.05). ERβb was also significantly higher in late sex changers than control females (p-value <0.01) as well as early sex changers (p value <0.01). ERβb mRNA abundances in sex-changing females reach levels approximately two and a half times those of non-changing females by stages 4-6 after TP male removal and initiation of sex change (fig 1.2c. ANOVA: $F_{2,19}$= 5.830, p< 0.0001; post hoc Tukey-Kramer HSD: Late sex changers > control females, p < 0.01; late sex changers> early sex changers, p<.0001).

Abundances of ERβa showed no statistically significant differences between sexual phenotypes (Fig 1.3b. ANOVA $F_{2,19}$= 5.830, p>0.05; posthoc Tukey-Kramer HSD: TP = females: p= 0.3109; TP = IP males, p=0.3109). The levels of ERβa also showed little variation over the course of sex change (Fig 1.2b, ANOVA: $F_{2,19}$= 5.830, p>.05; post hoc Tukey-Kramer HSD: Late sex changers = control females, p=0.4753; late sex changers=early sex changers, p=0.771). We divided the study into two groups- the sexual phenotype comparison consisted of untagged fish while the sex changers comparison were only tagged fish that had been observed.

Discussion

Estrogen receptor mRNAs exhibit differential expression both between the sexes and over the course of sex change in the bluehead wrasse brain. While several studies have addressed ER expression in the brain of teleosts (croaker, Hawkins et al., 2000, 2005, zebrafish, Menuet et al 2004) and a few have investigated sex differences (medaka, Hiraki et al 2011, *Halichoeres*, Kim et al2002), no study to date has looked at the potential role all three ERs may play in sex change processes. Evidence from other teleosts indicates gonadal status may strongly affect estrogen receptor expression. For example, the overall expression levels of ER mRNAs in the medaka brain were correlated with phenotypic sex and not with genetic sex. Expression of ER mRNAs was strongly responsive to steroid hormone environment, indicating that the sex differences are not attributable to sex chromosomes effects (Hiraki et al 2011).

While estrogens play a key stimulatory role in producing male-typical neuronal and behavioral phenotypes in mammals (reviewed in Balthazart et al, 2009, Trainor et al 2006), the opposite may be true for many fishes. One example is the bluebanded goby whose brain aromatase activity declines rapidly at the onset of female-to-male sex change (Black et al., 2005; Pradhan et al., 2015) while another is the plainfin midshipman whose hindbrain aromatase activity is much higher in females and female-mimic males than in large territorial males (Forlano et al, 2005, Schlinger et al. 1999). Studies of sex changing species indicate E_2 administration inhibits female-to-male sex change and/or male-typical behaviors (reviewed by Godwin, 2010). Reductions in estrogen signaling also appear important for sex change in bluehead wrasses, brain aromatase decreases rapidly during the early stages (1-3) of sex change (Lamm et al, unpublished). Interestingly the increase in relative abundances of ERα and ERβb mRNAs do not appear until the later stages (4-6) of sex change (Fig 1.2). 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. In Medaka, steroid receptor mRNA levels were positively correlated with circulating steroid levels in males, but negatively correlated in females, suggesting different regulatory control between sexes (Hiraki et al, 2012). It is possible there is a similar transition in regulatory control during sex change in bluehead wrasses.

The estrogen receptor's main mechanism of action is as a transcription factor through binding to the estrogen response element of target genes upon activation by estrogen and then recruiting coactivator proteins, which are responsible for target gene transcription (Menuet et al 2004). Other ERs may associate with membrane proteins and can be rapidly activated by estrogen exposure to cells. As stated earlier, ERs have a well conserved DNA binding domain (DBD), a variable N-terminal, a flexible hinge and a C-terminal ligand binding domain (LBD) (NCBI).

The comparisons of the bluehead wrasse nucleotide sequences for the DBD and LBD of ERα, ERβa, and ERβb with ER sequences in four other fish species as well as humans showed a very high degree of sequence conservation with other ERs in these two conserved domains of the molecule (Table 1.2). In all the species compared the ERβas share a high degree of sequence similarity with other ERs in the conserved domains of the molecule. However, there are several significant amino acid changes in these domains that are shared by all of the cloned ERβas (Hawkins et al, 2000 and 2004). These substituted amino acids may have functional significance that distinguishes ERβa from ERα and ERβb. Many of these diagnostic residues are located near or within regions involved in ligand binding in mammalian ERs (Ekena et al 1997, Brzozowski 1997, Shiau 1998). Erβa amino acid substitutions have led to changes in ligand preferences and affinities and these changes may reflect distinct physiological functions for ERβa (Hawkins et al 2000). Previous findings have also indicated that ERα and ERβb, while acting separately or cooperatively on specific gene targets, are positive transcriptional regulators of estrogen action, but the role of ERβa is unclear (Griffin et al 2013). Our results indicate ERβa abundance does not show significant differences across sexual phenotypes or sex change (at least at the whole forebrain level), which is different than the findings for ERα and ERβb. The lack of observed difference for ERβa appears generally consistent with the studies cited above suggesting a differing physiological role for this ER.

We did find an increase in ERα and ERβb over the course of sex change in the bluehead wrasse (Figs. 1.2a and 1.2c). Estrogen receptor 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 the 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 capacity and could be upregulated due to low ambient estradiol levels. In contrast to these results, a more recent study in *A. burtoni* found little difference between ERβa and ERβb in various brain regions between subordinate and dominant social groups, while levels of ERα did differ (Maruska et al, 2013). In *A. burtoni* circulating E_2 and ERα mRNA levels increased in the brains of ascending males suggesting that increased estrogen sensitivity in specific social nuclei may be important for the expression of behaviors (territorial and/or reproductive) and physiological changes that happen during social transition to dominance (Huffman et al, 2012, O'Connell and Hoffman, 2012).

Griffin and coworkers (2013) found that estrogen regulation of gene expression is specific to the ER subtype and target gene. Interactions involving ERα and one or both of the ERβs are already operative in early development. For example, in zebrafish embryos, a knockout of ERβb blocked induction of cytochrome P450 aromatase B mRNA (Griffin et al, 2013). Estrogen receptor knockout studies have also indicated important roles for estrogen signaling in social behaviors. Deletion of the ERα gene in mice decreases male aggression, causes deficits in social recognition tasks and inhibits sexual behaviors (Ogawa et al, 2009, Imwalle et al 2002, Ogawa et al, 1997).

Our results for ERα and ERβb are consistent with the findings that the expression of ERα, ERβa and ERβb in most fish tissues is significantly different in females than in males (Socorro et al., 2000, Choi and Habibi, 2003, Hawkins et al., 2005 and Lynn et al., 2008). In contrast, the three spot wrasse *Halichoeres trimaculatus* showed no significant differences in levels of ERα mRNA expression across sexual phenotype (Kim et al, 2002). The timing of expression and levels of each ER mRNAs can also differ during early development and gonadal growth as they likely have different roles in sex differentiation and gonadal development (Halm et al., 2004, Tingaud-Sequeira et al., 2004, Greytak and Callard, 2007 and Pikulkaew et al., 2007). Our results also show that ER mRNAs are differentially expressed over the course of sex change. The different ER subtypes are also differentially sensitive to estrogen and estrogen-mimicking chemicals found in the environment. For example, ERα expression is more sensitive to estrogen stimulation than either ERβa or ERβb (Urushitani et al., 2003, Menuet et al., 2004, Greytak and Callard, 2007 and Nelson and Habibi, 2010, Unal, 2014).

Previous studies analyzing the location of the specific ER mRNAs in the brain showed that ERα is well expressed in the classical neuroendocrine regions of the brain such as the preoptic area and the mediobasal and caudal hypothalamus, whereas the ERβs have a wider distribution especially along the brain ventricles of the telencephalon and diencephalon (Munchrath et al, 2010, Coumailleau et al, 2015). ERβa and ERβb mRNAs were also expressed in the preoptic area and other parts of the hypothalamus, but in much lower abundances than ERα. Munchrath and coworkers (2010) found that the distribution of the ERβb mRNA subtype is more widespread than ERβa. ERα is expressed primarily in neurons, in particular in dopaminergic neurons of the preoptic region (Linard,1998), GABA neurons (Anglade, 1999), GnRH 3 neurons (Zempo, 2013) and kisspeptin neurons (Zempo,2013; Mitani, 2010; Escobar, 2013). A recent study in *A. burtoni* also found that ERα is located within the lateral portion of the dorsal telencephalon (D1) and the dorsal and caudal regions of the ventral telencephalon (Vd and Vc) and the brainstem regions including the periaqueductal gray (Munchrath et al, 2010).

In future studies, microdissection should be used focusing on the forebrain hypothalamus and telencephalic regions along with the POA. Studies from *A. burtoni*, as well as other teleosts, suggest that all ER subtypes are expressed in parts of the preoptic area and hypothalamus (Harbott *et al.*,2007; Hawkins et al., 2000 and 2005; Menuet et al., 2002;

Hiraki et al., 2012). In *A*. burtoni there were robust effects of differences in reproductive capacity ER mRNA expression in the anterior, but not middle portions of the brain. The anterior portion consisted primarily of the telencephalon which is important in sensory and cognitive processes (Burmeister et al, 2007). The hypothalamus is known to express high levels of steroid hormone receptors and is likely to be a central player in generating behavioral and physiological responses to changes in steroid hormone levels. In Medaka, ERα and ERβb mRNAs were found in higher concentrations in the preoptic area in females. This may be in contrast with our results as the POA showed a significantly lower abundance of ERs mRNAs in male medaka, potentially indicating these nuclei represent female specific target sites for estrogen in that species.

The value of a microdissection is also seen in a recent study by Maruska and colleagues (2013). A previous study in *A. burton*i showed that social dominance influenced estrogen receptor mRNA levels in the brain such that dominant males had higher levels of multiple receptor subtypes (ERβa and ERβb) primarily in the forebrain (entire telencephalon and portion of anterior POA) as compared to subordinate males (Burmeister, 2007). However, the later study used microdissection to increase the neuroanatomical resolution in the forebrain and it was discovered that there were relatively few cases where dominant males had higher mRNA levels of any estrogen receptor compared to subordinates (Maruska, 2013). Restricting sampling to certain areas of the brain in bluehead wrasses would provide a more detailed picture of changes in ER mRNA expression between sexual phenotypes and across sex change. Unfortunately, this approach was not sufficiently developed for bluehead wrasses when these studies were conducted.

This study documents significant differences in abundances of ERα and ERβb mRNA at the whole forebrain level both across sexual phenotypes and with sex change while. differences were not found for ERβa. What remains unknown, however, is how changes in these estrogen receptors might influence the transcription of specific downstream genes to alter neuronal plasticity and function leading to physiological and behavioral change. While future studies are needed to fully understand the neuroendocrine plasticity that characterizes responses to social opportunity in *T. bifasciatum,* the results presented here build on previous studies to suggest alterations in estrogen signaling in the brain are likely to be key regulators of this process.

Table 1.1 qPCR primers for ERα, ERβa, ERβb, and EF1α

Primer	Forward	Reverse	Amplicon Size
Erα	GGA GAC CTT GTC CCC ACA AC	CCT CGG CTC TCT TCG GGA TA	81 bp
Erβa	CTC CGC AGA CGA TGT GGT AA	GGA GAG GAC GCA ACT TCC AA	129 bp
Erβb	GCA GCC TCT AGG CTA CAA CG	CGA GGG GTT CAC ACC ATT CA	148 bp
EF1α	ATC GGC GGT ATT GGA ACT GT	CGA CCA TAC CGG GCT TCA	67 bp

Species	ERα DBD	ERα LBD	ERβa DBD	ERβa LBD	Erβb DBD	Erβb LBD
Larimichthys crocea, Large Yellow Croaker	91%	96%	95%	86%	100%	100%
Halichoeres tenuispinis, Chinese Wrasse	97%	96%	95%	87%	100%	93%
Danio rerio, Zebrafish	85%	83%	97%	84%	97%	78%
Micropogonias undulatus, Atlantic Crroaker	89%	96%	95%	86%	100%	99%
Homo sapiens, Humans	97%	61%	n/a	n/a	96%	64%

Table 1.2. Sequence Identity (amino acid comparisons) between the Bluehead ERs and other species within the highly conserved DNA-binding domain (DBD) and ligand-binding domain (LBD)

```
Era       -------------MLLRQSPEQSRQTCGPAPRPRISSALSELETLSPQHPSPPQRGPHGG
ErBa      -MAVATYPEKDQPLLQLQKVDSSRVGSCVLS---------------P-VLSSP--SETSQ
ErBb      MASSPGLDSDPLPLLQLQEVDSSKAMERPSS-----------PSLLPAVYSPPR-GMDSH
                       :*  *. :.*:                      *   * *  .  .

Era       DMYPEESRGSGGVGTVDFLEGTYDYAAPTPAQTPLYSLSTPGYYSAALDTHGQPSDSSIQ
ErBa      TICIP----------SPYSDLSHDFT-----TIPFYSPS--------IFSYAAPGISDCP
ErBb      TVYIP----------SPYTDNNHEYNHGSGGPMSFYSPS--------VLSYARPSATDSP
           :               : : .:::         :** *        : ::. *. :.

Era       SLGSGPSSPLVFVPSSPRLSPF-M--HLPS---HHYLETSSTPVYRSSVSSSQQSISREE
ErBa      S-VHRSISPSLFWPSHSHVGPS-IPLHHSQTRPQHSQPIQSPSQWVELSQIDA-----VI
ErBb      SSLCGPLSPSAFWPPHSHSNLPSLTLRCPQP--LGYNEPGLHAPWIESKSHSIIGSSSII
          *      **  * *   :     :  :  .              : .  . .

Era       HCGTSDESYSMGESGAGA--VAGCFEMAKEMRYCAVCSDYASGYHYGVWSCEGCKAFFKR
ErBa      STSKSARRRSL-----ENEDEVVSSGGKADLHYCAVCHDYASGYHYGVWSCEGCKAFFKR
ErBb      GCNKPPGKRSEGGVNGVNPSLCSSVVGKADMHFCAVCHDYASGYHYGVWSCEGCKAFFKR
             .     *             .     ::::**** **********************

Era       SIQGHNDYMCPATNQCTIDRNRRKSCQACRLRKCYEVGMMKGGVRKDRGRVLRRDKRRTG
ErBa      SIQRHNDYICPATNQCTIDKNRRKSCQACRLRKCYEVGMTKCGIRKERGTYRTPQTRRIS
ErBb      SIQGHNDYICPATNQCTIDKNRRKSCQACRLRKCYEVGMMKCGVRRERCSYRGTRHRRGG
          *** ****:**********:******************* * *:*::*        ** .

Era       MSDKDKGSKDREQRTVPPQGRRKHSSSVGGG-----------KPPVISMPPDQVLLLLQG
ErBa      RPSSQSSTND--PAGL------------K---RPAG--VLLNLSHPAVLTPEQLIERIME
ErBb      LQPRDPTGRGLVRVGLGSRAQ--RHLHLEGPLTPVTPLPQMNHVHHATMSPEEFISRIME
              :   .      :                                : *::.:  :

Era       AEPPILCSRQKLSRPYTEVTMMTLLTSMADRELVHMIAWAKKLPGFLQLTLHDQVQLLES
ErBa      AEPPEIYLMKDMKRPLTEANIMMSLTNLADKELVHMISWAKKIPGFVELSLVDQVHLLEC
ErBb      AEPPEIYLMEELKKPFTEASMMMSLTNLADKELVLMISWAKKIPGFIELCLADQIHLLKC
          **** :   :.:.:* **..:*  **.:**:*** **:****:***::* * **::**:.

Era       SWLEVLMIGLIWRSIHCPGKLIFAQDLILDRSEGDCVEGMAEIFDMLLATTSRFRMLKLK
ErBa      CWLEVLMVGLMWRSVDHPGKLIFSPDLSLSREEGSCVQGFSEIFDMLVAATSRVRELKLQ
ErBb      CWLEILMLGLMWRSVDHPGKLIFSPDFKLNREEGQCVEGIMEIFDMLLAATSRFRELKLQ
          .***:**:**:***:. ******: *: *.*.**.**:*: ******:*:***.* ***:

Era       PEEFVCLKAIILLNSGAFSFCTGTMEPLHDGVAVQNMLDIITDALIHHISQSGCSAHQQS
ErBa      REEYVCLKAMILLNSNLCTSSPQTEEELESRNKLLRLLDSVIDALVWAISKLGLSTQQQT
ErBb      REEYVCLKAMILLNSNMCLGSSETNEELQSRSKLLRLLDAVTDALVWAIAKTGLTFRQQY
           **:*****:*****     .  * * *..   : .:** : ***:  *:: * : :**

Era       RRQAQLLLLLSHIRHMSNKGMEHLYSMKCKNKVPLYDLLLEMLDAHRLHRPDRPAESWSQ
ErBa      LRLGHLTMLLSHIRHVSNKGM---------------------------------------
ErBb      TRLAHLLMLLSHIRHVSNKGMDHLHCMKMKNMVPLYDLLLEMLDAHIMHSSRLPRRAPQQ
           *  .:* :*******:*****
```

Figure 1.1 The deduced amino acid sequences of the three bluehead cDNAs [Bluehead wrasse ER α (bhERα), Bluehead wrasse ER βb (bhERβb), Bluehead wrasse ER βa (bhERβa)] indicating diagnostic features of ER genesBluehead sequences were aligned with 15 other ERs by using clustal omega online program. The DBD is underlined and the LBD is red. The transactivation function region, TAF-2, which is involved in ligand-dependent transactivation, is underlined in red. Sequence notation includes alignment gaps (–) and shared identity with all three (*)

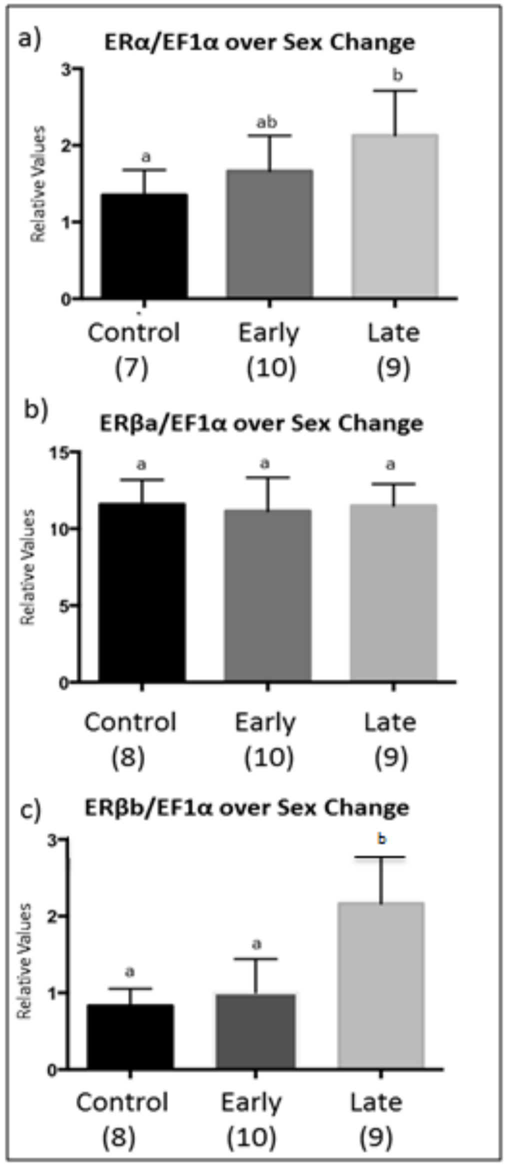

Fig1.2 a-c:. Normalized values of full brain Er mRNA expression in different stages of sex change (control=females, early= stages 1-3, late= stages 4-6) in the bluehead wrasse. A. Erα B. Erβa C. ErBb Values are mean ± 1 SEM.

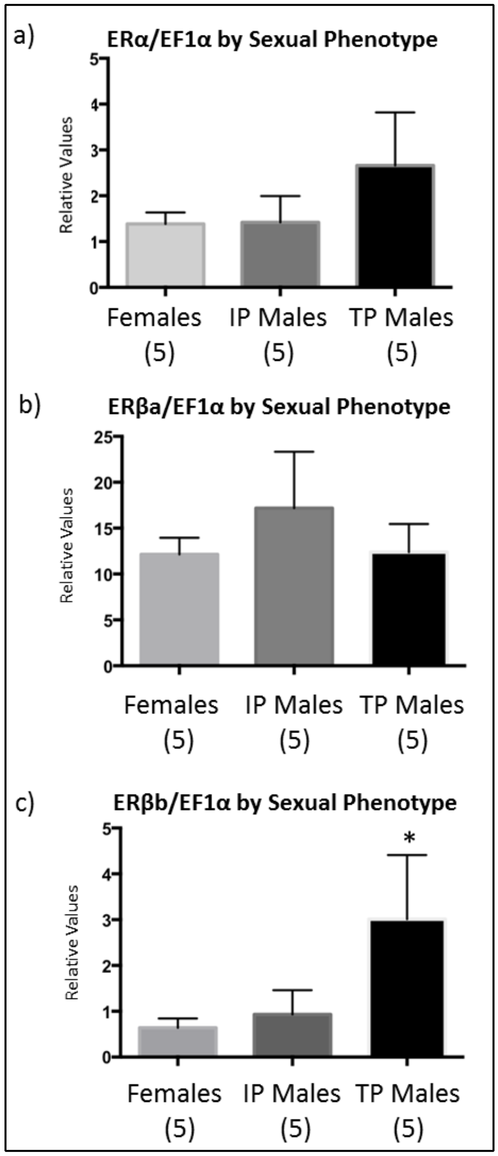

Fig. 1.3 a-c: Normalized values of full brain Er mRNA expression in different sexual phenotypes of the bluehead wrasse- female, IP males, and TP males. a) ERα b) ERβa c) ERBb. Values are mean ± 1 SEM.

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