ABSTRACT

MARSH, KRISTEN ERICA. Neuroendocrine Transduction of Social Cues in the Bluehead Wrasse, *Thalassoma bifasciatum*. (Under the direction of Dr. John Godwin.)

Understanding behavioral adaptations to changing social conditions is a key challenge for social neuroscience. The bluehead wrasse, *Thalassoma bifasciatum*, is a protogynous sex-changing fish with sex and role change under social control, making it an excellent system in which to study neuroendocrine mechanisms regulating male sociosexual behavior. The behavioral changes associated with a change in social dominance in bluehead wrasses are dramatic, take place rapidly, and can be studied in the full complexity of the natural environment.

Sex hormones play key roles in neural modulation of behavioral processes. Because of the likelihood of complex interplay between estrogens and androgens in the brain, steroidogenic enzymes have received much attention recently and this is especially true of the enzyme aromatase. Aromatase is responsible for the conversion of androgens into estrogens and is strongly implicated in the development and regulation of sexual behaviors in many species. In this project, we have exploited aromatase's key role in regulating the reproductive neuroendocrine axis in order to begin investigating how changes in local estrogen synthesis are involved in the neural modifications that lead to the assumption of male-typical sexual behavior. The following studies address the neuroanatomical potential for interactions, the existence of neural sex differences in distribution, and finally the consequences of estrogen manipulations on the behavioral and neural phenotypes.

We used immunocytochemistry (ICC) to characterize distributions of aromatase-immunoreactive (ir) cells in the brain of the bluehead wrasse and to examine their

relationship with AVT-ir neurons and tyrosine hydroxylase-ir (TH-ir) neurons in key sensory and integrative areas. Aromatase-ir appeared to be expressed in glial cell populations in the dorsal and ventral telencephalon, the preoptic area of the hypothalamus, and the lateral recess of the third ventricle among other brain areas. Aromatase-ir fibers are closely associated with AVT-ir neurons throughout the preoptic area and were also co-regionalized with TH-ir neurons, indicating the potential for functional interactions.

We then examined levels of brain aromatase mRNA across phenotypes of the bluehead wrasse using oligonucleotide *in situ* hybridization to assess sexual dimorphisms in expression. Aromatase mRNA abundances in the preoptic area of the hypothalamus were significantly higher in females compared than either initial phase (IP) males or terminal phase (TP) males. Levels in IP males were also two-fold higher than aromatase levels in TP males.

Finally, in field experiments involving gonadally-intact and gonadectomized females, we found that estradiol (E_2) implants prevented behavioral sex change in large females after removal of the dominant males. By contrast, cholesterol-implanted control females showed full behavioral sex change, along with a higher frequency of aggressive interactions and male-typical courtship displays than E_2-implanted animals. Following the field studies, we assessed relative abundances of aromatase mRNA and found greater expression in the POA of E_2-implanted females than in cholesterol-implanted controls in gonadally-intact females.

The findings presented here are consistent with a model of estrogenic inhibition of male-typical behavior and behavioral sex change in the bluehead wrasse. Supporting evidence includes the distribution of aromatase-ir cells in brain nuclei important in neural control of behavior, differences in aromatase expression across sexual phenotype, and the

prevention of behavioral sex change by estrogen implants coupled with increased mRNA abundances. We propose that decreases in neural estrogen synthesis are responsible for the expression of male-typical sexual behavior in the bluehead wrasse. This research provides a base from which we can further explore the functional relationship between neuroendocrine traits and complex behavioral shifts in this model, offering insights into social influences on the development and maintenance of sociosexual behaviors more generally.

Neuroendocrine Transduction of Social Cues
in the Bluehead Wrasse, *Thalassoma bifasciatum*

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DEDICATION

To my mother, Joan Elizabeth Shelton Marsh. Thank you for giving me the imagination and the strength to make it this far. There is not one of my friends who does not know that a true map of my world has you at its center.

BIOGRAPHY

Kristen Erica Marsh grew up in the small town of Humboldt, TN, where she became interested in freshwater ecology and ichthyology after a failed goldfish freedom experiment. Eschewing the advice of her high school counselor, she decided to pursue a degree in biology rather than joining the U.S. Coast Guard. She received her B.A. from The University of Mississippi in Oxford, MS, mentored by Dr. Glenn Parsons, shark biologist. After graduation, Erica worked for two years as a Senior Research Technician in the Environmental Toxicology Research Program (Department of Pharmacology, The University of Mississippi) directing aquatic experiments and fieldwork operations for three separate laboratories. Her M.Sc. degree in Biology focused on waterborne pheromones in the control of sex change in marine gobies and was completed in the Bahamas and Puerto Rico under Dr. Marc Slattery, marine chemical ecologist (The University of Mississippi).

In 2003, Erica came to North Carolina State University to pursue a doctoral degree in behavioral neuroendocrinology under the mentorship of Dr. John Godwin. Upon completion of her degree, she plans to continue in a postdoctoral research position in the field, while working towards broadening her focus to include human clinical psychology.

ACKNOWLEDGMENTS

I would like to thank Dr. John Godwin for his mentorship over the past four and a half years. It has been a struggle against the currents, and I am grateful that he has encouraged me throughout both the exciting discoveries and the disappointments. I would also like to thank my committee members, Drs. Christina Grozinger, Jane Lubischer, Stephen Nowicki, and Coby Schal. All of you have helped to better my dissertation, both the writing and the research itself. Thank you for your insightful commentary and most of all, your time. To Dr. Robert Anholt, and all members of the W.M. Keck Center for Behavioral Biology, thank you for the support you have provided, both in funding and in intellectual resources. The interdisciplinary perspectives and after-hours discussions have been invaluable.

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I would like to thank my family, for believing in me and supporting my seemingly never-ending pursuit of higher education. The friendships that I have made here in Raleigh are some of the strongest I have ever experienced, and I am deeply grateful to them for

providing strength, wisdom, and laughter throughout these years. I would especially like to thank Alexis Edwards, Rebecca Bartel, Christina Strom, Susan Pendse, and Laura Shewmon, for their patience, humor, and the all-important Girls' Nights, whether going out or staying in. Finally, I would like to thank my fiancé, Justin M. Hunkin, with all my heart. You have been a quiet strength and I am profoundly indebted to you. There is no life I can imagine without you that would be so full of laughter and joy.

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CHAPTER I

Recognizing the context of a social interaction and generating the appropriate response is essential for critical functions such as reproduction, parental care and kin recognition. There is considerable inter- and intra-sexual variation across vertebrate taxa in the complexity of phenotypic variation within an environment. We can understand species specific behavioral adaptations by exploring the mechanisms by which individuals respond to social situations. The neural and hormonal processes subserving these behaviors are likely to be highly conserved, since these are basic life processes common across taxa (Insel and Fernald, 2004). Comparative studies in animals may help identify the neural substrates of social behavior (Barr et al., 2003, Burmeister, Jarvis and Fernald, 2005, Goodson et al., 2005, Goodson, 2005), and the mechanisms underlying behavioral adaptations to changing social conditions which have not yet been comprehensively identified. Understanding the molecular basis of this transduction of social information, and the neuropeptide pathways that lead to the appropriate behavioral, physiological, and cellular changes is a key challenge for social neuroscience. More generally, social interactions are known to be important to human health. Insights into social influences on neural function are becoming increasingly important in order to understand the role social behavior plays in human mental illnesses such as affective and autism spectrum disorders (Caspi et al., 2003, Lesch, 2007, Lim, Bielsky and Young, 2005, Lord et al., 2000).

What pathways might be involved in the neural control of behavioral adaptations? Understanding the mechanisms behind these changes in response to social interactions requires a model organism exhibiting a complex social system that can be manipulated in a

context relevant to the natural behavior of the animal, with quantifiable changes in behavior.

The natural life-history characteristics of the bluehead wrasse, *Thalassoma bifasciatum*, provide such a model. The bluehead wrasse is a protogynous (i.e. female-to-male sex changing fish that also exhibits two discrete, alternate male phenotypes. In this species, sex and role change is under social control, with females changing into males after becoming socially dominant. When the TP male is removed from a social group, the largest female or IP male present will change sex and/or role to become a TP male (Warner and Swearer, 1991). Gonadal change takes place over 8-10 days, but male-typical behavior is often exhibited within minutes of removal of the TP male by sex-changing females. Importantly, females that have been ovariectomized nevertheless undergo sex change when made socially dominant (by removal of dominant TP males), effectively 'decoupling' the brain from gonadal influences (Godwin, Crews and Warner, 1996). These traits make the bluehead wrasse an excellent system in which to study the neuroendocrine mechanisms regulating sociosexual behavior. The behavioral changes associated with a change in social dominance are dramatic, take place rapidly, and can be manipulated in the full complexity of the natural environment.

What neuroendocrine pathways are the most likely 'suspects' in the control of sexual and aggressive behavior? Steroid hormones regulate the expression of male-typical sexual behavior in all vertebrate species (De Vries and Simerly, 2002). Sex steroids act both genomically and non-genomically in the brain to coordinate the production of these behaviors and appropriate physiological changes, including the expression of secondary sexual characteristics. Recent studies have emphasized the key role of estrogen, rather than

testosterone, in the control of male sexual behavior, because many of the effects of testosterone come about after metabolic conversion to estradiol via the enzyme aromatase (Balthazart and Ball, 1998). Estrogen critically affects sexual differentiation and the display of male sexual behavior in many vertebrates, including mice (Hull and Dominguez, 2007, Roselli, Abdelgadir and Resko, 1997), quail (Ball and Balthazart, 2004, Balthazart and Foidart, 1993), and frogs (Moore, Boyd and Kelley, 2005, Sassoon and Kelley, 1986). These hormonal effects are typically thought to work relatively slowly through receptors that are ligand-activated transcription factors. While classical pathways are clearly important, increasing evidence also points to a role for rapid actions of steroid hormones (Balthazart, Baillien and Ball, 2001, Remage-Healey and Bass, 2004, Remage-Healey and Bass, 2006, Revankar et al., 2005). Production of neurosteroids, specifically local estradiol synthesis by aromatization of testosterone, has been shown to regulate the reproductive axis, act in the control of sexual behavior, and regulate neural plasticity (Lephart, Lund and Horvath, 2001). Neurosteroid production can also be rapidly altered. Changes in aromatase activity in the quail brain can be seen within minutes of pharmacological manipulation and are reversible, suggesting physiological events could potentially regulate estrogen production in the brain over short time courses (Balthazart et al., 2003, Balthazart, Baillien and Ball, 2001).

In the following studies, I have used aromatase as a molecular marker to explore the potential role of neurally-produced estrogen in the behavioral sex change process and, more generally, to better understand species-specific behavioral adaptations and phenotypic plasticity in social situations. The experiments that follow combine both laboratory procedures examining the neuroanatomical potential for a role of neural estrogen production

in behavioral adaptation and field manipulations utilizing the clear social hierarchies bluehead wrasses form on the reef.

In Chapter II, I describe immunocytochemical characterization of the distributions of aromatase-immunoreactive (ir) cells in the brain of the bluehead wrasse, and their relationship with arginine vasotocin-ir neurons and tyrosine hydroxylase-ir neurons in key sensory and integrative areas. Understanding the possible interaction of neural estrogen production with signaling systems such as the neuropeptide arginine vasotocin and the dopaminergic system is the first step in determining mechanisms that may underlie the behavioral effects. In Chapter III, I examine aromatase messenger-RNA (mRNA) levels across phenotypes of the bluehead wrasse using an oligonucleotide-based *in situ* hybridization assay. Phenotype differences in aromatase expression underscore the potential role(s) of neurally-synthesized estrogen in behavioral phenotype expression. A comparison of these sexual role dimorphisms in aromatase expression across vertebrate species may further our knowledge of the neuroendocrine cascades that control alternative reproductive behaviors.

Chapter IV describes field experiments utilizing estrogen implants in gonadally-intact and gonadectomized females in social environments that are permissive for sex change. The results are consistent with a model in which estrogen exposure is the critical determinant of the expression or inhibition of male-typical sexual and aggressive behaviors. Following the field studies, I assessed mRNA abundances of aromatase in both the gonadally-intact and gonadectomized animals implanted with either estradiol or cholesterol to examine neural correlates of the observed behavioral effects. In Chapter V, I summarize our contributions to

understanding the role of estrogen in the regulation of sexual behavior in this model species. The evidence presented, including the distribution of aromatase-ir cells in brain nuclei important in control of sexual behavior, phenotypic differences in aromatase expression, and increased mRNA abundances coupled with non-dominant behavioral phenotypes, suggests that neurally-produced estradiol is critical to the regulation of behavioral sex change. This research provides a base from which we can further explore the functional relationship between neuroendocrine traits and complex behavioral shifts, offering insight into social influences on the development and maintenance of sociosexual behavior.

Chapters II, III, and IV were written for separate publication and have either already been published or are currently being submitted for publication. As such, the chapters will be presented as either reprints or manuscripts, with stylistic differences intact according to the requirements of the respective journals.

CHAPTER II

AROMATASE IMMUNOREACTIVITY IN THE BLUEHEAD WRASSE, THALASSOMA BIFASCIATUM: IMMUNOLOCALIZATION AND CO-REGIONALIZATION WITH ARGININE VASOTOCIN AND TYROSINE HYDROXYLASE

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Aromatase immunoreactivity in the bluehead wrasse brain, *Thalassoma bifasciatum*: Immunolocalization and co-regionalization with arginine vasotocin and tyrosine hydroxylase

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Abstract

Sex steroid hormones regulate various neural functions that regulate vertebrate sociosexual behavior. A number of sex steroids can be synthesized de novo in the brain, including estrogens by the enzyme aromatase. Aromatase, the neuropeptides arginine vasotocin/vasopressin, and the monoamine neurotransmitter dopamine have all been implicated in the control of male sexual and aggressive behavior in a variety of vertebrates. This study examined the expression of brain aromatase in a teleost fish, the bluehead wrasse (*Thalassoma bifasciatum*), a teleost fish that exhibits socially-controlled behavioral and gonadal sex change. We used immunocytochemistry (ICC) to characterize distributions of aromatase-immunoreactive (ir) cells, and to examine their relationship with AVT-ir neurons, and tyrosine hydroxylase-ir (TH-ir) neurons in the key sensory and integrative areas of the brain of this species. Aromatase-ir appeared to be in glial cell populations, and was found in the dorsal and ventral telencephalon, the preoptic area of the hypothalamus, and the lateral recess of the third ventricle, among other brain areas. Aromatase-ir fibers are closely associated with AVT-ir neurons throughout the preoptic area, indicating the potential for functional interactions. Aromatase-ir cell bodies and fibers were also co-regionalized with TH-ir neurons, suggesting possible interaction between the dopaminergic system and neural estrogen production. The presence of aromatase in brain regions important in the regulation of sexual and aggressive behavior suggests local estrogen synthesis could regulate sex change through effects on signaling systems that subserve reproductive behavior and function.

Section: Cognitive and Behavioral Neuroscience
Keywords: aromatase; arginine vasotocin; dopamine; estrogen; teleost; hypothalamus

1. Introduction

In all vertebrate taxa including mammals, mating behavior is a complex sequence of behavioral responses requiring the ability to integrate endogenous hormonal and neurochemical changes with environmental information. The most important environmental information for many species can come from conspecifics. These social signals are often sexual in nature and have profound effects on both neural function and behavioral profiles. The mechanisms underlying behavioral adaptations to changing social conditions have not been comprehensively identified as yet and understanding the molecular basis of this transduction of social information is a key challenge for social neuroscience.

Sex hormones play key roles in neural modulation of behavioral processes. Both testosterone (T) and estradiol 17β (E_2) stimulate male sexual behavior in a variety of vertebrates (Cross and Roselli 1999). While 'classical' genomic pathways are clearly important for many of these effects, increasing evidence also points to rapid steroid actions on neurons and in the mediation of sexual behavior (Revankar et al. 2005; Remage-Healey and Bass 2004;). For example, changes in the conversion of androgens to estrogens by aromatase can be seen within minutes in the quail brain. This suggests estrogen production in the brain could potentially be regulated over short time courses and such rapid alterations would be consistent with observed estrogen effects on behavior (Balthazart et al. 2001, Balthazart and Ball, 2006). Rapid alterations in neural estrogen production have also been documented in sex changing fishes (see below).

We are focusing on modulation of neural estrogen through aromatase as a potential mechanism underlying rapid adaptation to changing social conditions in a sex changing coral reef fish, the bluehead wrasse (*Thalassoma bifasciatum*). Several studies have highlighted the importance of estrogens and the aromatase pathway in the gonadal sex change processes (Cardwell and Liley 1991; Cochran and Grier 1991; Godwin and Thomas 1993; Chang et al. 1994, 1995; Kroon and Liley 2000; Kroon et al. 2003) and have suggested that it is a decrease in E_2 levels that permits male development (Kroon et al. 2005). Similarly, we found that when the estrogen synthesis blocker 1,4,6-androstatrien-3,17-dione (ATD) is given alone or coadministered with T, complete color and gonadal sex change is induced in female *T. bifasciatum* (Austin et al. unpublished).

Many sex-changing fishes, including the bluehead wrasse, show very rapid behavioral changes during the sex change process. Male behaviors are often expressed within minutes or hours as an individual assumes social dominance (Robertson 1972; Warner and Swearer 1991; Godwin et al. 1996; Black et al. 2005). This short time scale of behavioral change is consistent with the rapid steroid actions on behavior in other systems discussed above. The neural form of aromatase (cytochrome P450b or AROb) is abundantly expressed in the brains of teleost fishes (Callard et al. 2001), including in key regions regulating sexual behavior (Schlinger et al., 1999; Forlano et al. 2001, 2005a,b; Chang et al. 2005; Kishida and Callard 2001; Menuet et al. 2003, 2005). As with the important role of aromatase in gonadal sex change processes, neural estrogen synthesis via AROb appears to be critical in transducing

social signals regulating male-typical sexual behavior under changing social conditions. In *Lythrypnus dalli*, another gobiid species with socially controlled sex change, neural aromatase activity in dominant females decreases significantly within hours of male removal (Black et al. 2005). This decrease in aromatase activity is correlated with rapid increases in aggressive and territorial behavior in the transitional females.

Other neural signaling systems have also been implicated in the process of behavioral and gonadal sex change. Arginine vasotocin (AVT), the neuropeptide found in fishes that is homologous to tetrapod arginine vasopressin (AVP), is of particular interest in the bluehead wrasse system. AVT affects reproductive behaviors in a broad range of vertebrates (Thompson and Moore 2003; Moore 1992; Moore and Lowry 1998; Goodson and Bass 2001), including several fishes (Salek et al., 2001; reviewed by Moore 1992; Moore and Lowry 1998; Goodson and Bass 2001). Expression of AVT is higher in dominant male bluehead wrasses than females and increases rapidly with sex change (Godwin et al., 2000). This increased expression of AVT is driven by social dominance and is independent of gonads (Semsar and Godwin 2003). Additionally, administration of AVT increases aggressive and courtship behavior typical of dominant males (Semsar et al. 2001) while an AVT receptor antagonist blocks territorial acquisition in large males and behavioral sex change in females (Semsar and Godwin 2004).

Monoamine neurotransmitters represent other neurochemical systems that can influence and be influenced by steroid hormone signaling. Behavioral studies in mice, quail, teleost fishes, and primates have demonstrated rapid steroid actions involving neurotransmitters (Hull et al. 2004). The dopamine system regulates sexual behavior and function in a variety of vertebrates and is often responsive to the steroid hormone environment (see Hull et al. 2004). Both human and non-human animal studies strongly suggest that dopamine facilitates male sexual behavior (Dominguez and Hull 2005). Levels of dopamine and other monoamines change over the course of female-to-male sex change in a congener of the bluehead wrasse (*Thalassoma duperrey*, Larson et al. 2003a) and manipulations of dopaminergic signaling can influence this process (Larson et al. 2003b). In quail, estradiol rapidly modulates male sexual behavior and this correlates with changes in levels of dopamine in the brain (Cornil et al. 2006). Dopamine has also been shown to down-regulate neural aromatase activity in quail (Balthazart et al. 2002; Balthazart and Ball, 2006), providing strong evidence for the involvement of monoamines in the neurochemical pathway controlling male sexual behavior.

The bluehead wrasse (*Thalassoma bifasciatum*) offers an experimentally tractable model in which to investigate the neurochemical pathways that underlie behavioral adaptation to changing social conditions. This common Caribbean reef fish has three sexual phenotypes: large, brightly colored terminal phase (TP) males and smaller, yellow and brown striped, initial phase (IP) males and females. TP males develop from either sex-changing females or role-changing IP males. Most TP males maintain territories on reefs and court and mate with females within these territories, although there is variation among males in these behaviors (see Semsar et al., 2001). Females display the yellow and brown coloration and live within groups on reefs that normally include a dominant TP male. IP males are non-territorial,

usually mate in large aggregations ('group spawns'), and may also mimic females to 'sneak' or 'streak' spawn with TP male/female pairs. When the TP males are removed from a social group, the largest female or IP male present will change sex and/or role to become a TP male (Warner and Swearer, 1991). Gonadal change takes place over 8-10 days, but male-typical behavior is often exhibited by sex changing females within minutes of removal of the TP male (Godwin et al. 1996).

This unpredictable social system requires rapid adaptation to changing dominance hierarchies. The first step in determining how estrogen might influence these behaviors and other neural signaling systems in the bluehead wrasse brain is an assessment of the sites of neural estrogen synthesis and neuroanatomical potential for interactions. Specifically, our objectives in this study are to: 1) examine the distribution of aromatase expression in the brain of the bluehead wrasse, and 2) assess the potential colocalization of aromatase with AVT and tyrosine hydroxylase to determine the neuroanatomical potential for functional interactions between these systems. Full descriptions of AVT and tyrosine hydroxylase distributions in the bluehead wrasse brain are beyond the scope of this study.

2. Results

Aromatase immunolocalization

Aromatase-labeled cell bodies were visualized throughout the brain and found in greatest abundance in the forebrain. The antibody used in this study was made against a conserved amino acid sequence of known teleost aromatases, and was first used in the plainfin midshipman (Forlano et al. 2001). An identical pattern of labeling was consistently seen in the same brain regions between animals. The antibody showed cytoplasmic labeling of aromatase-ir cell bodies and also labeled fibers. In control assays, omission of the primary antibody eliminated labeling throughout the brain, and serial dilutions of the primary antibody produced progressive decreases in labeling. In general, aromatase-ir distribution was similar to distribution patterns seen in other teleosts (Fig. 1). These distributions are described below for bluehead wrasses. The morphology of the aromatase-ir cells suggests that aromatase expression in the bluehead wrasse brain is of glial origin, and this is consistent with that seen in other teleosts (Forlano et al. 2001, Menuet et al. 2003, 2005). No differences in distribution of labeling were observed between estrogen-implanted fish and wild-caught fish.

Aromatase-ir was observed along nearly all ventricular surfaces in the brain. Aromatase-ir labeling in the telencephalon was seen along the lateral margins of both hemispheres, and medially along the walls of the telencephalic ventricle (Fig. 2A) and the third ventricle in the diencephalon. Aromatase positive cells were also found along the intermediate zone and postcommissural nucleus of the ventral telencephalon (Fig. 2B), along the ventricle in the habenula, and in the suprachiasmatic nucleus (SCN). In the preoptic area of the hypothalamus, aromatase-ir populations were found in the anterior parvocellular preoptic nucleus (PPa), the posterior parvocellular preoptic nucleus (PPp), and both the magnocellular and gigantocellular preoptic nuclei (Fig. 2C). Further caudally in the diencephalon, cells

Fig 1

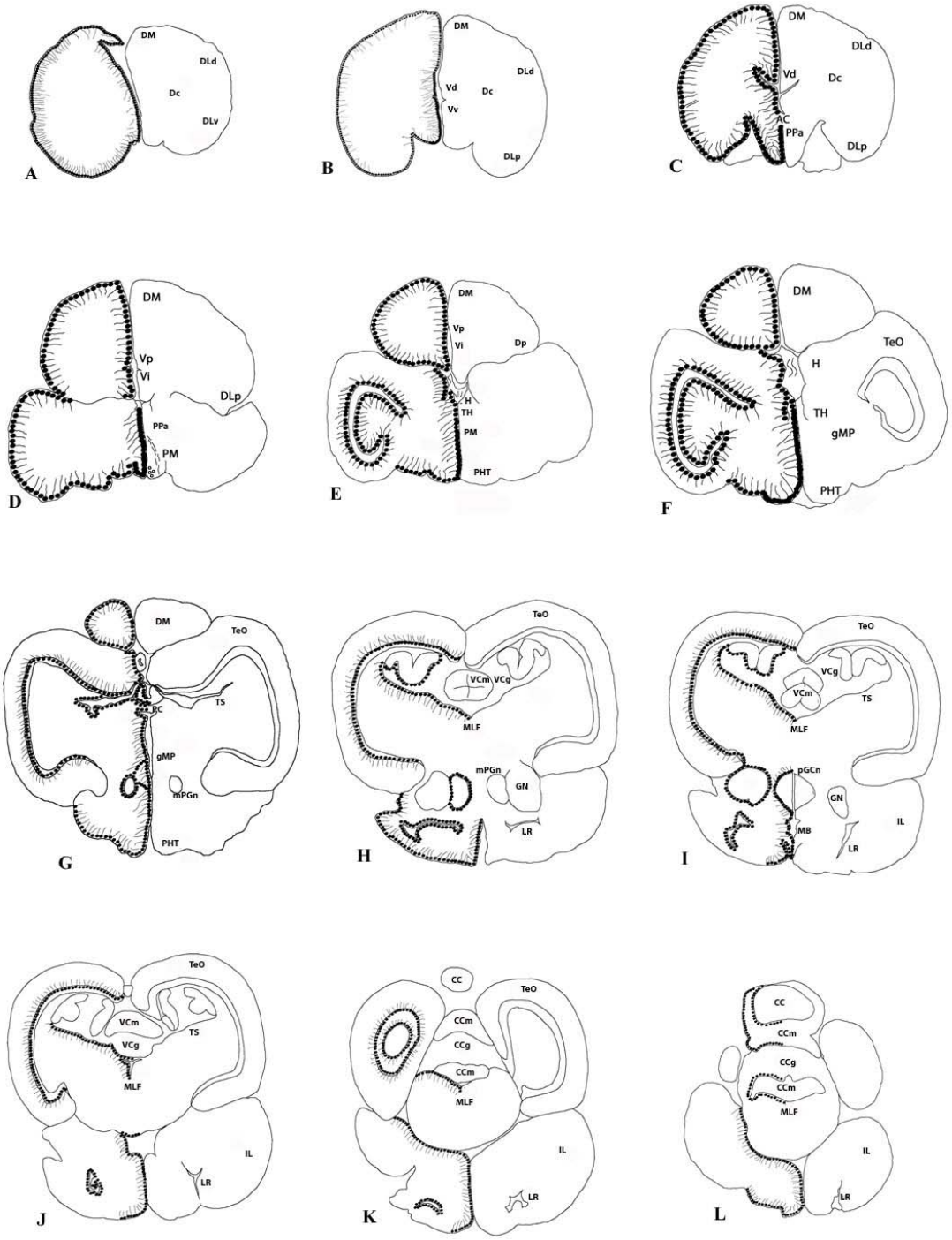
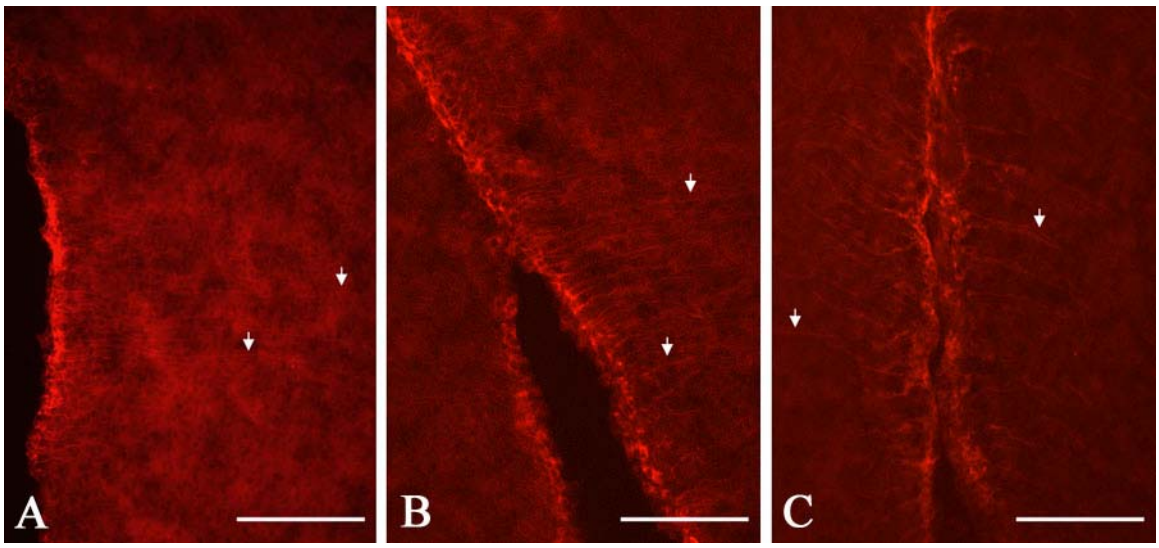

A
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DLp
C
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PPa
DLp
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DM
Vp
Vi
DLp
PPa
PM
E
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Vp
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TeO
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gMP
mPGn
PHT
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TeO
VCm
VCg
MLF
mPGn
GN
LR
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TeO
VCg
VCm
TS
MLF
pGCn
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VCm
TS
VCg
MLF
IL
LR
K
CC
TeO
CCm
CCg
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CC
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CCm
MLF
IL
LR

Fig. 2



were labeled in both the lateral and anterior tuberal nuclei and in the dorsal hypothalamus (specifically the posterior periventricular nucleus or NPPv).

In the midbrain, aromatase labeling was found in several areas. In the optic tectum, aromatase-ir was seen along the internal edge in the stratum periventriculare, with long processes extending outwards dorsally and laterally, suggesting that the cells are radial glia (Fig. 3A). In other areas of the midbrain, aromatase-ir cells are found in the periventricular gray zone, in the vicinity of the posterior commissure, and both the torus longitudinalis and torus semicircularis. Aromatase-ir cells also line the ventricular wall on the medial side of the thalamus and are found in the preopticohypophysial tract and tectal ventricle.

In the hindbrain, lower levels of aromatase-ir were apparent. Cells were found lining the fourth ventricle and cerebral aqueduct (CA) and lateral recess, as well as the medial longitudinal fasciculus (MLF) and inferior lobe of the hypothalamus. In the cerebellum, aromatase-ir populations were seen both above the molecular cell layer and throughout the granule cell layer (Fig. 3B-C).

Aromatase co-regionalization with AVT and Tyrosine Hydroxylase
Consistent with a previous study in our laboratory (Semsar and Godwin 2003), AVT-ir cells were found in three distinct populations: the parvocellular, magnocellular, and gigantocellular preoptic nuclei. Aromatase-ir cells and fibers in these regions were found in very close association with AVT-ir neurons (Fig. 4A-B). Tyrosine hydroxylase-ir fibers were also co-regionalized with AVT-ir neurons in the gigantocellular, and magnocellular preoptic areas (Fig. 4C-D). The source of these TH fibers is uncertain, but strongly staining TH-ir cell bodies are found in the posterior parvocellular preoptic area, just ventral to the magnocellular AVT-ir neuron populations. More generally, tyrosine hydroxylase-ir labeled fibers were found in areas that also showed aromatase-ir cells, including the dorsal and ventral telencephalon, the anterior and posterior parvocellular preoptic nucleus (Fig. 5A), the optic tectum and the torus semicircularis (Fig. 5B). Tyrosine hydroxylase staining in the stratum periventriculare (SPV) of the optic tectum was verified with a single fluorescent label (Fig. 5C) that was absent when the primary antibody was eliminated (Fig. 5D).

Discussion

In this study, we have demonstrated widespread localization of aromatase immunoreactivity in the bluehead wrasse brain. The distribution pattern was generally similar to that of aromatase-ir cells described for other teleost species (Gelinas and Callard, 1997; Forlano et al., 2001; Menuet et al. 2003, 2005, Goto-Kazeto
et al. 2004, Strobl-Mazzulla et al. 2005). Importantly, aromatase-ir populations are seen in key brain areas associated with the integration of social cues and with the regulation of male-typical sociosexual behavior. Below, we compare the distribution of aromatase-ir with that described from other teleosts and consider potential functional interactions between aromatase-expressing glia and AVT and dopaminergic neurons.

Fig. 3

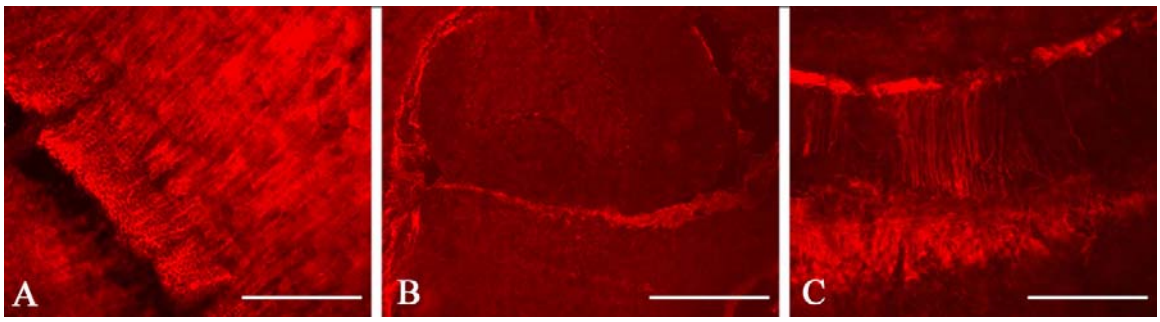


Fig. 4

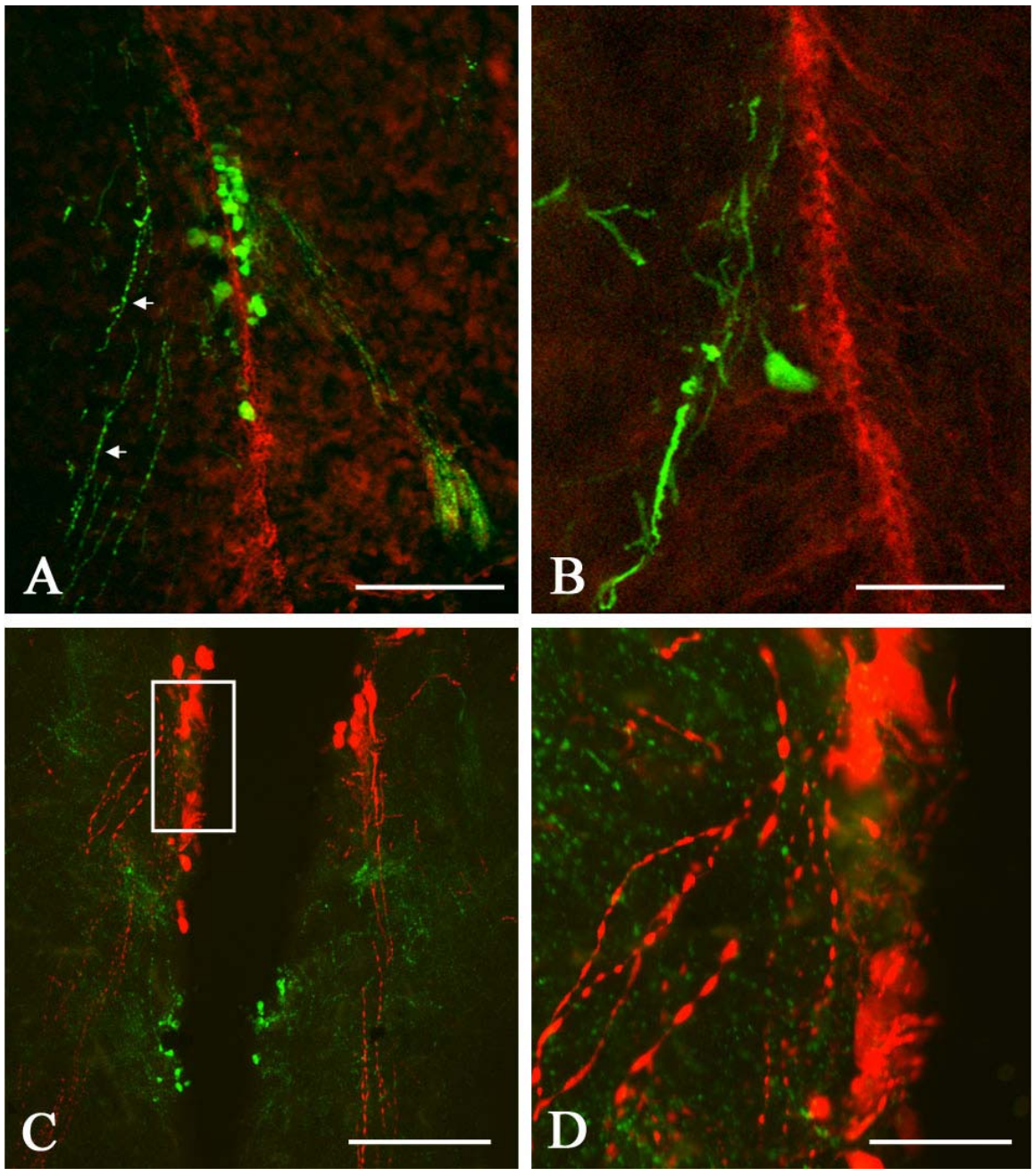
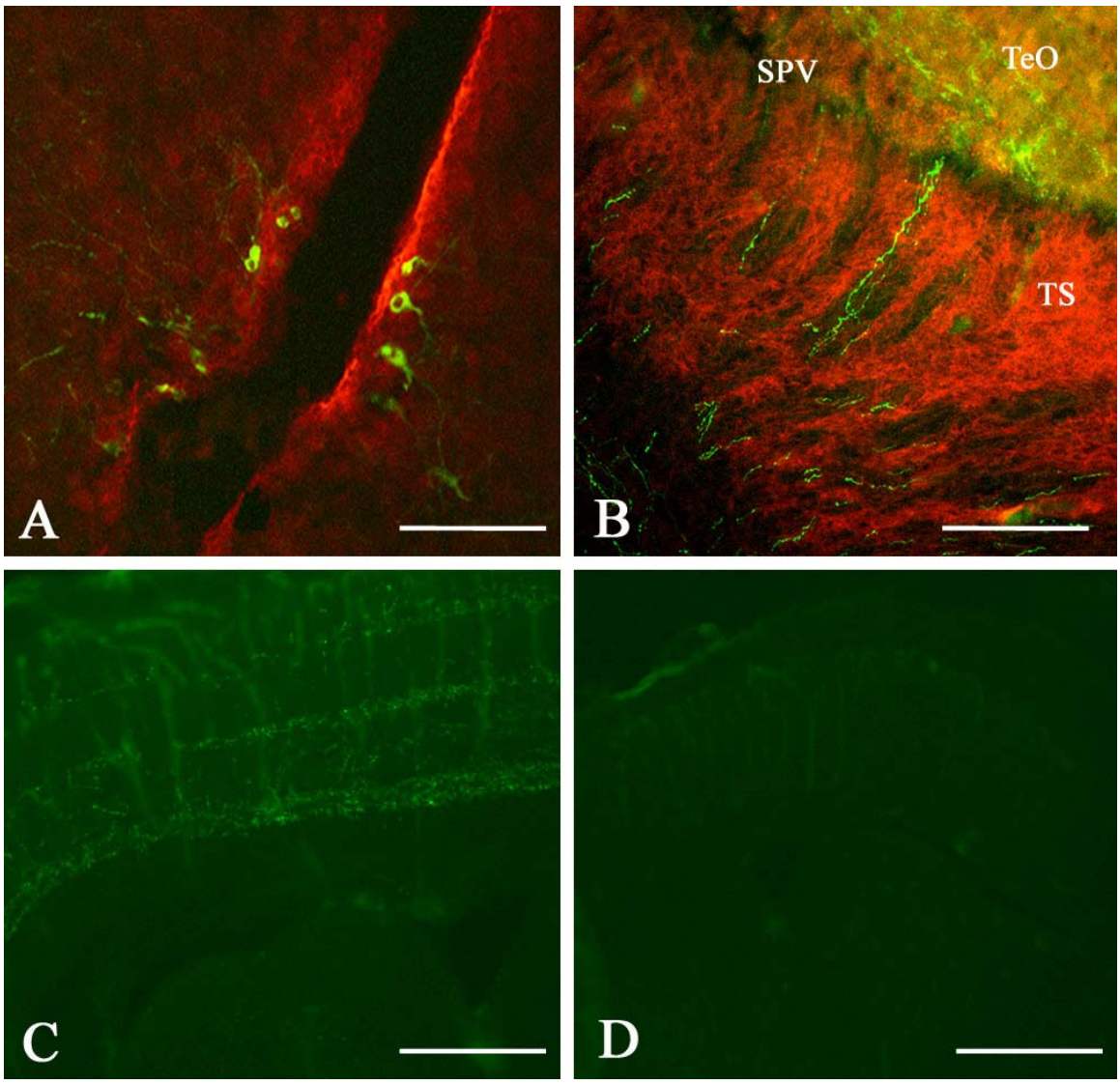


Fig. 5



Aromatase Distribution: Comparisons Across Species

The pattern of aromatase-ir found here for bluehead wrasses is generally consistent with that described for other teleosts, but there are some interesting differences. The similarities include labeling in the preoptic and ventral areas of the telencephalon which are important integrative areas for social behavior and reproductive function. These ventral telencephalic nuclei are considered homologous to amygdalar and septal areas in mammals and other tetrapods (Wullimann and Mueller 2004). Two major differences seen among the teleost species that have been investigated is the expression pattern of aromatase found in the hindbrain and in the optic tectum. The plainfin midshipman (*Porichthys notatus*) shows strong expression in the hindbrain, especially in the sonic motor nucleus (SMN), but not in the optic tectum. The plainfin midshipman is a vocalizing species and aromatase activity in the hindbrain differs across sexual morphs that vary in vocalizing behavior in this species (Schlinger et al. 1999). Hindbrain aromatase expression has not been well characterized in a species where communication is primarily based on visual cues instead. In contrast to the midshipman, aromatase-ir cells were abundant in several layers of the optic tectum in the bluehead wrasse. Both rainbow trout and zebrafish also show aromatase-ir cells in the optic tectum (Menuet et al. 2003, 2005). As with bluehead wrasses, these are species that rely more on visual cues in their environment. Callard and coworkers (2001) reported aromatase mRNA and protein in the goldfish (*Carassius auratus*) retina and optic tectum and suggested neurally-derived estrogen may modulate the visual system.

Potential interactions with the AVT and Dopamine systems

From studies in our laboratory on sites of AVT production in the bluehead wrasse brain, we have located three populations of AVT neurons within the preoptic area (parvo-, magno-, and gigantocellular; Semsar and Godwin 2003), as well as one population in the ventral tuberal hypothalamus (Elkins and Godwin, unpublished). Immunocytochemical labeling in this study is consistent with these findings, and shows co-regionalization of AVT-ir neurons in the preoptic area with aromatase-ir cells. Based on cell morphology these aromatase-ir cells appear to be glial. This finding is in contrast with findings from other vertebrates, in which aromatase is normally expressed neurons and in glia only after brain injury. However, aromatase expression in glia is consistent with results in the plainfin midshipman based on both morphology and GFAP labeling (*Porichthys notatus;* Forlano et al. 2001) and other teleost species based on either labeling with glial markers or on cellular morphology (rainbow trout and zebrafish, Menuet et al. 2003, 2005; Pellegrini et al. 2005).

Distributions of aromatase-ir cells also overlapped with both fibers and somata of TH-ir neurons. The distribution pattern of TH-ir staining in the bluehead wrasse is consistent with that seen in several other species of teleosts including the zebrafish, Senegalese sole, rainbow trout, and goldfish (Rink and Wullimann, 2002, Rodriguez-Gomez et al. 2000, Vetillard et al. 2002, Hornby et al. 1987). Most notably, TH-ir cells and fibers in the bluehead wrasse were found in areas that also show aromatase-ir cell populations, including in the dorsal and ventral telencephalon and the preoptic area. We tentatively interpret these findings to indicate dopaminergic rather than noradrenergic innervation in these regions based on

findings in zebrafish (Rink and Wullimann 2002) and the lack of staining found using a dopamine β-hydroxylase antibody in these areas of the bluehead wrasse brain (unpublished data). The co-regionalization of aromatase-ir cells and TH-ir neurons suggests dopamine signaling could affect or be affected by neural estrogen synthesis, particularly in the preoptic area. Dopamine plays an important role in regulating aromatase expression and/or activity in the vertebrate brain and this interaction is particularly well studied in the Japanese quail (for reviews, see Balthazart et al. 2002; Balthazart and Ball 2006).

Neural Aromatase and Sex Change

We postulate that neural estrogen synthesis via aromatase plays a critical role in regulating sex change in the bluehead wrasse. Specifically, we hypothesize that neurally-produced estrogen blocks behavioral sex change under socially inhibitory conditions as recently proposed for a sex changing goby (Black et al., 2005). Aromatase localization in brain regions that show intrasexual variation in AVT expression could allow transduction of social cues and initiation of sex change through modifications in estrogen signaling. We further propose that this effect is at least partly mediated through interactions with the AVT system and possibly the dopaminergic system. This pathway would be consistent with evidence of a key role for neurally produced estrogen affecting sexual differentiation and function in a variety of vertebrates including fishes (Black et al. 2005) and with recent findings regarding estrogen signaling and AVP neuron function in mammals (Plumari et al. 2002). An alternative model for steroid hormone regulation of behavioral sex change focused on the potential role of corticosteroids and inhibitory effects on AVT neurons has been proposed and is described in detail by Perry and Grober (2003).

Blocking aromatase activity with inhibitors can induce gonadal sex change under inhibitory social conditions (Kroon and Liley 2000; Kroon et al., 2005; Bhandari et al. 2004) and this is also true in the bluehead wrasse (Austin et al., unpublished). However, it is not clear whether these effects of aromatase inhibition on gonadal sex change are mediated in the gonads or the brain. However, patterns in other species suggest neural aromatization has important effects on behavior. The plainfin midshipman (*Porichthys notatus*) exhibits two distinct male phenotypes that show pronounced differences in behavior which are correlated with differences in aromatase expression in various brain regions involved in sexual and aggressive behavior (Schlinger et al. 1999; Forlano et al. 2001; Forlano et al., 2005a, b). Recently, Black and coworkers (2005) documented similar sex differences in neural aromatase activity in the female-to-male sex changing goby *Lythrypnus dalli* and found that neural aromatase activity rapidly declined at the onset of socially induced sex change.

Estrogen (E_2) critically affects sexual differentiation and the display of male sexual behavior in many vertebrates. However, there is considerable diversity in the nature of these effects both among species and in terms of their biochemical mediation. The best understood example of estrogen effects on male reproductive behavior is seen in rodents where estrogen plays a critical role in both the organization during development and activation in adulthood

of male sexual behavior (Meisel and Sachs 1994). However, not all systems are masculinized by treatment with estrogen and estrogen effects on male-typical sexual behavior can be very rapid. Estradiol masculinizes the vasotocin system of Japanese quail during development (Panzica et al., 1998) and can influence male sexual behavior rapidly in adulthood in mice, quail, and the plainfin midshipman (Cross and Roselli 1999; Balthazart and Ball 2006, Remage-Healey and Bass 2004). These rapid effects suggest estrogen actions may occur non-genomically, consistent with other rapid estrogen effects documented in neurons (for review, see Balthazart and Ball 2006). The estrogens active in the brain may be derived from either gonadal sources or local conversion through neural aromatization.

How might rapid alterations in aromatase activity occur? We examined the dopaminergic system here in addition to the vasotocin system in part because of recent findings in Japanese quail (*Coturnix japonica*) showing that aromatase activity can be decreased within minutes by calcium-dependent phosphorylation processes (Balthazart et al. 2001, reviewed in Balthazart and Ball, 2006). Based on dopaminergic innervation, the presence of DA receptors in the preoptic area, and induction of changes in aromatase activity through activation of DA receptors, these authors suggest that dopaminergic input to aromatase-positive neurons (Cornil et al. 2004) rapidly affects estrogen production in the preoptic area in response to environmental cues. No comparable information is available for fishes, but dopamine is a key regulator of reproductive processes in some species via its inhibitory effects on gonadotropins (Devlin and Nagahama 2002). Extensive TH-ir innervation of the preoptic area, as found here, has also been found in other species of fishes (e.g., Vetillard et al. 2002). Interestingly, these TH-ir neurons also express estrogen receptors (ERs) in the rainbow trout (*Oncorhyncus mykiss*; Linard et al. 1996).

The close association of aromatase-ir and AVT-ir cells in the bluehead wrasse preoptic area suggests the possibility of direct estrogen effects on AVT signaling. In mice, ERβ has been co-localized with AVP in the paraventricular nucleus (PVN), the putative homologue of the magnocellular preoptic area in teleosts (Hrabovszky et al. 1998; Nomura et al. 2002; Kapsimali et al. 2001) and ERβ regulates AVP expression in the mouse PVN (Nomura et al. 2002). Several studies in fishes show expression of at least one ER subtype in areas with aromatase-ir cells including the preoptic region (fishes have three ER subtypes ERa, ERβa, and ERβb; Hawkins et al., 2001, 2005; Forlano et al. 2005c2005a,b; Menuet et al. 2003; Tchoudakova and coworkers, 1999). Large neurons in the preoptic area of the Atlantic croaker express an ERβ subtype (ERβb; Hawkins et al. 2001, 2005). The neurochemical phenotype of these neurons is not yet characterized, but their size and location are consistent with that of AVT and isotocin neurons. If some of these Erβb-expressing neurons are AVT neurons, it would suggest possible direct estrogenic regulation of AVT similar to the situation with AVP in mammals.

Here we demonstrate the close association of aromatase-ir, AVT-ir, and TH-ir cells in areas of the brain known to regulate male sexual behavior and reproductive function. These findings suggest modulation of neural estrogen synthesis through aromatase expression and/or activity could mediate rapid behavioral adaptation to changing social conditions in the

bluehead wrasse. Consistent with this hypothesis, we have recently found that estrogen implants can block behavioral sex change under socially permissive conditions (unpublished data). In order to further investigate estrogenic regulation of sociosexual behavior in this species, our future goals are to compare aromatase mRNA expression across phenotypes of the bluehead wrasse and over the course of sex change and explore the potential role of estrogen receptor subtypes (ERα, βa, and βb) in mediating the behavioral effects of estrogens.

Experimental Procedures

All experimental methods described here were approved by and are in compliance with the guidelines of the Institutional Animal Care and Use Committee of North Carolina State University (NCSU).

For hormone administration, female and IP male bluehead wrasses were obtained from (a commercial dealer (FL), shipped to NCSU, and held in 107 l glass aquaria on a recirculating seawater system while being fed daily with commercial flake food.. After a two day acclimation period, surgeries were performed as in previous studies (Godwin et al., 1996; Semsar and Godwin 2003, 2004; Austin et al. in prep). Fish were implanted abdominally with 8mm Silastic implants (Silastic tubing, 1.47 mm ID, 1.96 mm OD, Dow Corning, Midland, MI; approximately 20ul total volume) containing either estradiol benzoate (1μg/g, n=2 of each phenotype) dissolved in peanut or peanut oil alone as a vehicle control (n=2 of each phenotype). Individuals were active and feeding the next day and grew new scales over the surgical wound within a week, suggesting full recovery. After 10 days, the fish were killed using an overdose of MS-222 (tricaine methanesulfonate, Sigma, St. Louis, MO) and their brains dissected out, fixed in 4% paraformaldehyde for 24 hours, then transferred to a 30% sucrose solution for cryoprotection for 24 hours, and sunk in freezing medium (OCT, TissueTek, Elkart, IN) for cryosectioning.

In order to assess potential effects of estradiol benzoate implants on aromatase-ir distributions, we also examined unmanipulated animals. We captured adult female, IP male, and TP male wrasses (n=5 each) directly from patch reefs in Teague Bay, St. Croix, U.S. Virgin Islands. The fish were captured by liftnet in June 2005 during daylight hours and were killed within 2 minutes of capture using an overdose of MS-222 (tricaine methanesulfonate, Sigma, St. Louis, MO). Their brains were processed as described above, and all cryoprotected brains were held at -80° C until cryosectioning.

Immunocytochemistry

For immunocytochemistry (ICC) analysis of aromatase-ir, AVT-ir, and TH-ir cells, brains were coronally cryosectioned into six adjacent series of 20 μm sections, meaning adjacent sections on a slide were separated by 100 μm, and then stored at -80° C until utilized in assays. Double-labeling of the selected antigens was performed simultaneously. Slides were allowed to come to room temperature (RT), dehydrated in a series of ethanol washes (70, 95,

100% EtOH; 1 minute each), air dried and outlined in PAP pen (Fisher Scientific, St. Louis, MO). The slides were then rinsed in phosphate-buffered saline (0.1M PBS; 2 X 10 minutes), blocked in 10% normal goat serum (NGS, Vector Laboratories, Burlingame, CA) in PBS (30 minutes, RT), and placed in primary antisera diluted in PBS containing 1% NGS (16-20 hours, RT). The antisera and working dilutions used were: aromatase, a rabbit polyclonal used at a dilution of 1:50 (Forlano et al. 2001); arginine vasopressin (AVP), a guinea pig polyclonal (1:1,000; DiaSorin, Stillwater, MN); tyrosine hydroxylase (TH), a mouse monoclonal (1:1000; Immunostar, Hudson, WI), and glial fibrillary acidic protein (GFAP), a guinea pig monoclonal (1:100; Molecular Probes, Portland, OR). The aromatase polyclonal antibody (kindly provided by A.H. Bass, Cornell University) was developed against a conserved amino acid sequence of known teleost aromatases and the specificity of labeling was confirmed using an antibody designed against a specific teleost aromatase within that group, the plainfin midshipman (*Porichthys notatus*; Forlano et al. 2001). All other antisera used in this study were obtained commercially. Following the 16-20 hour incubations of the slides in the primary antisera, sections were rinsed in PBS (2 X 10 minutes) and placed in fluorescent secondary antibodies (both 1:100, Alexa-fluor 594 nm for anti-aromatase, 488nm for both anti-AVT and anti-TH; Molecular Probes, Portland, OR). After a 2 hour incubation in the dark (RT), sections were rinsed in PBS (2 X 10 minutes), allowed to air dry in the dark, and coverslipped using Gel Mount (Fisher Scientific, St. Louis, MO). Elimination and serial dilutions of the primary antibody from incubation were used as controls. Fluorescent images were taken using a Leica DMR digital microscope (Leica Microsystems, Bannockburn, IL) with a Hamamatsu Orca-ER cooled CCD camera (Hamamatsu Corporation, Hamamatsu City, Japan) and Openlab 3.5.1 software (Improvision, Inc., Lexington, MA). The images were sharpened and adjusted for light and contrast in Adobe Photoshop 7.0 (Adobe Systems, San Jose, CA). Neuroanatomical nomenclature follows that of Wullimann et al. (1996).

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Figure Legends

Figure 1. Diagram of neuroanatomical distribution of aromatase-ir positive structures on representative coronal sections (A–L) of the brain of the bluehead wrasse. Relative numbers, sizes, and locations of aromatase-ir cells and fibers are on the left side of the midline, with structures labeled on the right. See abbreviations in Table 1.

Figure 2. Aromatase immunoreactivity in the telencephalon and diencephalon. All images are fluorescently labeled with anti-aromatase in red. Arrows point to long fibers that suggest labeled cells are radial glia. Panel A, lining the telencephalic ventricle in the medial zone of the dorsal telencephalic area; B, postcommissural nucleus of ventral telencephalon; C, magnocellular preoptic area. Scale bars: 200μm.

Figure 3. Aromatase-immunoreactivity in the optic tectum and granule cell layer of the valvula of the cerebellum. All images are of aromatase immunoreactivity fluorescently labeled with anti-aromatase in red. Panel A, cell bodies in the stratum periventriculare (SPV) with fibers extending dorsally through the optic tectum (TeO); B, cell bodies and fibers in the granule cell layer of the valvula of the cerebellum; C, closeup of lower central portion of B. Scale bars: A and C, 100μm; B, 400 μm.

Figure 4. Arginine vasotocin-immunoreactivity co-regionalized with aromatase-immunoreactivity and with tyrosine hydroxylase-immunoreactivity. Panels A and B show anti-aromatase in red and anti-arginine vasopressin in green. Panel A, magnocellular preoptic area and preoptico-hypophysial tract; B, gigantocellular preoptic area. The arrows in A show 'beading' of arginine vasotocin-immunoreactive fibers. Panels C and D show anti-arginine vasopressin in red and anti-tyrosine hydroxylase in green. Panel C, arginine vasotocin-ir neurons in magnocellular preoptic (PM) area and TH-ir neurons ventral to the PM; D, inset of C showing TH-ir fibers co-regionalized with arginine vasotocin-ir neurons. Scale bars: A, 400μm; B, 100μm; C 200μm; D, 50 μm.

Figure 5. Aromatase-immunoreactivity (red) co-regionalized with tyrosine hydroxylase-immunoreactivity (green) in the top panel, and tyrosine hydroxylase-immunoreactivity alone (green) in the bottom panel. Panel A, posterior parvocellular preoptic nucleus; B, aromatase-ir fibers in the torus semicircularis (TS) and anti-tyrosine hydroxylase cell bodies in the stratum periventriculare (SPV) with fibers extending ventrally through the TS; C, anti-tyrosine hydroxylase cell bodies in the SPV with fibers extending dorsally into the TeO; D, absence of primary antibody eliminates staining in section adjacent to C. Scale bars: A, 200 μm; B-D, 100 μm.

Table 1. Abbreviations

AC	anterior commissure
CC	crista cerebellaris
CCg	granule cell layer of the cerebellum
CCm	molecular cell layer of the cerebellum
DC	central nucleus of telencephalic area dorsalis
DLp	posterior part of lateral zone of telencephalic area dorsalis
DLd+v	dorsal and ventral division of lateral zone of area dorsalis
DM	medial zone of telencephalic area dorsalis
DP	posterior zone of telencephalic area dorsalis
gMP	gigantocellular portion of the magnocellular preoptic nucleus
Gn	glomerular nucleus
H	habenula
IL	inferior lobe of the hypothalamus
LR	lateral recess
MB	mammillary body
MLF	medial longitudinal fascicle
mPGn	medial preglomerular nucleus
TeO	optic tectum
PC	posterior commissure
PHT	preopticohypophysial tract
PGCn	preglomerular commissural nucleus
PM	magnocellular preoptic nucleus
PPa	anterior parvocellular preoptic nucleus
PPp	posterior parvocellular preoptic nucleus
Th	thalamus
TLo	torus longitudinalis
TS	torus semicircularis
VCg	granule cell layer of the valvula cerebelli
VCm	molecular cell layer of the valvula cerebelli
Vd	dorsal zone of telencephalic area ventralis
Vi	intermediate zone of telencephalic area ventralis
Vp	postcommissural nucleus of telencephalic area ventralis
Vv	ventral zone of telencephalic area ventralis

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CHAPTER III

PHENOTYPIC DIFFERENCES IN AROMATASE MRNA ABUNDANCE IN A TELEOST FISH

This paper is in preparation for submission to the Journal of Neuroscience:

Marsh, K. Erica, Hawkins, M. Beth, and Godwin, John. Phenotypic differences in aromatase mRNA abundance in a teleost fish.

Phenotypic differences in aromatase mRNA abundance in a teleost fish

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Abstract

Many species exhibit alternative reproductive behaviors, providing opportunities to explore the neurophysiological underpinnings of behavioral adaptation. The bluehead wrasse is a teleost fish that exhibits socially-controlled sex change, with two discrete, alternate male phenotypes. The behavioral transformations in a female or subordinate male that are associated with a change in social dominance are dramatic and occur within minutes of removal of the dominant male. It is well known that estrogens can critically affect the display of reproductive behavior in many species. The enzyme aromatase, which converts testosterone to estradiol, may be a key player in regulating the expression of behavioral phenotypes in the bluehead wrasse. Here we cloned a portion of the brain type aromatase gene (CYP19A2) of the bluehead wrasse. The 416bp sequence showed higher percentage identity with other teleost brain aromatases (81-91%) than to other teleost ovarian aromatases (67-71%). Levels of brain aromatase mRNA were assessed across phenotypes of the bluehead wrasse using *in situ* hybridization. Aromatase mRNA abundances in the preoptic area of the hypothalamus were significantly higher in females compared with either initial phase (IP) males or terminal phase (TP) males. There was no significant difference between IP males and TP males. These findings are consistent with neural estrogen synthesis through aromatization, and further underscore estrogen's key role in the neural control pathway of these discrete behavioral phenotypes.

Introduction

Alternative reproductive phenotypes are products of the interaction between environmentally-based selection pressures, hormonal pathways and genetic influences. Understanding the role of neuroendocrine mechanisms in producing these alternative phenotypes will provide insights into the physiological bases underlying individual variation in behavior and production of complex phenotypic traits more generally. Examples of discrete alternative 'morphs' are present in many vertebrates (Knapp, 2004, Moore, et al. 1998, Moore,1991, Remage-Healey and Bass, 2007) with teleost fishes providing a wide array of alternate reproductive phenotypes that differ in morphology, physiology, and endocrine tactics. The bluehead wrasse, *Thalassoma bifasciatum*, is one such example and presents an experimentally tractable model in which to further explore links between phenotypic changes that appear to be hormonally regulated and steroid signaling in the brain.

The bluehead wrasse is a protogynous hermaphrodite common throughout the Caribbean Sea. The species exhibits three distinct morphological phenotypes: large, bright blue and green, terminal phase (TP) males; smaller, yellow and brown striped, initial phase (IP) males and females. TP males develop from either sex-changing females or role-changing IP males. Territorial TP males maintain territories on reefs and court and mate with females ('pair spawns') within these territories. IP males are non-territorial, usually mate in large, undefended aggregations ('group spawns'), and may also attempt to gain fertilizations by 'sneak' or 'streak' spawning with spawning pairs of TP males and females. Females are non-territorial, typically feed in groups for most of the day, and spawn every 1-3 days. During the daily spawning period, a female will move to spawning sites and either pair-

spawn with a TP male or mate with IP males at a group spawn.

When the TP male is removed from a social group, the largest female or IP male present will change sex and/or role to become a TP male (Warner and Swearer, 1991). Gonadal change takes place over 8-10 days (Warner and Swearer, 1991), but male-typical behavior is often exhibited by sex changing females within minutes of removal of the TP male. Interestingly, females that have been ovariectomized nevertheless undergo behavioral sex change when made socially dominant (by removal of dominant TP males) (Godwin, Crews and Warner, 1996). This decoupling of behavioral and gonadal change provides an excellent system for examining social influences on neuroendocrine signaling. Another key aspect of this system is that the morph changes occur rapidly in the animal's adult life, suggesting an equally rapid change in endocrine signaling in order for an animal to perform the appropriate behavioral responses. This 'activational' change will allow for detection of the functional relationship between the neuroendocrine traits and complex behavioral shifts, providing great insight into social influences on neural function.

Steroid hormones are involved in regulating the expression of sex-specific reproductive behavior in all vertebrates (De Vries and Simerly, 2002). Many studies have focused on the role of estrogen in controlling male sexual behavior, as many of the neural effects of testosterone occur through its conversion in the brain to estradiol by aromatase (Balthazart and Ball, 1998). This is true organizationally in mice and rats, where aromatized estrogen is responsible for both masculinization and defeminization of neural circuitry underlying sexual behaviors in the male brain (Arnold et al.,1984; Beach et al.,1969; McEwen et al.,1983; Morris et al.,2004); in primates, where restoration of adult copulatory

behavior after castration requires aromatized estrogen (Resko et al., 2000); and also in songbirds, where estrogen masculinizes the avian songbird pathway (Holloway & Clayton 2001). Recently, studies have focused on the more rapid and dramatic effects of estrogen on reproductive behavior that may be due to non-classical, non-genomic mechanisms of action (Moore, 1999; Remage-Healey et al., 2004; Revankar et al., 2005). Systemic estrogen injections cause rapid increases in both chemoinvestigatory and mounting behaviors in male rats within 15-35 mins (Cross and Roselli, 1999) and increase copulatory behavior in male Japanese quail within 15 mins (*Coturnix japonica*, Cornil et al., 2006). Production of neurosteroids can also be rapidly altered. Changes in aromatase activity in the quail brain can be seen within minutes of pharmacological manipulation and are reversible, suggesting neurophysiological events could potentially regulate estrogen production in the brain over short time courses (Balthazart et al., 2001; Balthazart et al., 2003; Balthazart et al., 2004).

Could modulation of aromatase activity be a mechanism underlying rapid adaptation to changing social conditions? In another teleost, neural aromatase is expressed in key regions involved in the control of sexual behavior (Forlano et al., 2001), possibly acting as a 'biochemical switch' mechanism regulating sexual phenotype development (Schlinger et al., 1999). In the blue-banded goby (*Lythrypnus dalli*), another fish with socially controlled sex change, aromatase activity in dominant females decreases significantly within hours of male removal (Black et al., 2005). This decrease in aromatase activity is correlated with rapid increases in aggressive and territorial behavior in the transitional female (Black et al., 2005). These findings suggest alterations in neural aromatization may function in the transduction of social signals regulating male-typical sexual behavior under changing social conditions.

Our aims in this study were to isolate and clone the bluehead wrasse brain aromatase (CYP19A2) and to investigate its expression levels across behavioral phenotypes of the bluehead wrasse. We previously characterized the distribution of aromatase immunoreactivity in the bluehead wrasse brain and potential interactions between aromatase-expressing glia and vasotocinergic- and tyrosine hydroxylase-expressing neurons (putatively dopaminergic, Marsh et al., 2006). The previous study establishes the neuroanatomical potential for a key role of aromatase activity in controlling the expression of male behavior. In this study, we test the prediction that aromatase expression differs across alternate behavioral phenotypes that vary in their expression of male-typical sexual and aggressive behaviors.

Materials and Methods

Study site and species: Adult bluehead wrasses of all three phenotypes were collected from patch reefs located near Glover's Reef Marine Research Station (Middle Caye, Glover's Reef, Belize) during February 2007. Females, initial phase (IP) males, and terminal phase (TP) males were captured using a lift net during the spawning period between 1030h-1400h each day in order to ensure accurate behavioral phenotyping. Fish were observed to determine baseline behavioral profiles, activity areas, and territorial status for TP males before capture per Semsar and Godwin 2003, 2004. Fish were sacrificed immediately on capture (within approximately two minutes on the boat) using an overdose of MS-222 (tricaine methanesulfonate), measured (standard length in mm) and their brains dissected out and fixed in 4% paraformaldehyde. After 24 hours fixation, brains were placed in 0.1M

phosphate buffer. After another 24 hours, brains were transferred to 30% sucrose in 0.1M phosphate buffer and held at 4° C until shipment to the laboratory at NCSU for processing. All experimental methods described here were approved by and are in compliance with the guidelines of the Institutional Animal Care and Use Committee of North Carolina State University (NCSU).

Cloning of bluehead aromatase cDNA: RNA was isolated from brain tissue from field-collected fish then reverse transcribed to cDNA using the OmniScript® RT kit (Qiagen, Valencia, CA) and Oligo(dT)15 primers (Promega, Madison, WI). The resulting cDNA was used as template in two polymerase chain reaction (PCR) amplifications of P450aromB. Two sets of nested degenerate oligonucleotide primers were designed based on an alignment of sequences of CYP19A2 genes from teleosts [*Carassius auratus* (GenBank accession number AB009335), *Danio rerio* (120031), *Oreochromis niloticus* (AF306786), *Porichthys notatus* (AF472578), and *Tilapia mossambica* (AF135850)]. In the first amplification, forward primer (5`-TCCKYYCTTCTTRGCAGG-3`) and reverse primer (5`-CCAGGATGGCCTTCATCATCA-3`) were predicted to amplify a 1211bp product from the consensus sequence. In the second amplification, nested primers (5`-GCCCACTACACMKCCAGATTT-3` and 5`-GCTGCTYTCTTRTGCTTG-3') were used to reduce amplification of undesired products and amplify a 416bp portion of P450aromB. This PCR product was cloned into pCRII (Invitrogen, Carlsbad, CA), transformed, amplified, purified and sequenced to confirm the identity of the clone as CYP19A2 using BLAST (NCBI). Two clones contained a sequence that matched other teleost aromatase CYP19A2

sequences. Recent alignments show a high degree of similarity between our partial brain aromatase sequence and those of other wrasses; sequences which were not available at the start of our project: *Pseudolabrus japonicus* (DQ298136.1), E value 5e-163 and *Halichoeres tenuispinis* (AY489060.1), E value 6e-156.

In situ hybridization: *In situ* hybridization was used to assess the relative abundance of brain aromatase mRNA as described previously for assessing mRNA abundances in bluehead wrasse brain tissue (Godwin et al., 2000; Semsar and Godwin, 2003) with some differences as described below. Brains were cryosectioned coronally into six adjacent series of 20 μm sections using a cryostat and held at -80°C until processed in assays. Three specific, non-degenerate, non-overlapping antisense oligonucleotide probes were designed against the 416 bp partial clone sequence of bluehead brain aromatase (5`-AAATAATCCCCTTCCCTTCCATCCCGATGCACTCCAGCCC-3`; 5`-CCACATGTCCAGAAGGATCGGTCATCTCTTTTAGGCGCTC-3`; and 5`-ATTAATCGGCACCCTGAGGAACAGCTTATTGGAGATGTCC). A mixture of equal amounts of these three oligonucleotides was end-labeled with ^{33}P-dATP (specific activity, 3000 Ci/mmol; Perkin Elmer, Boston, MA), added to hybridization buffer, and applied to slides as described previously (see Godwin et al. 2000; probes were added at 2 x 10^6 DPM/oligonucleotide probe for a total 6 x 10^6 DPM/slide). Following washes, dry slides were placed against GP phosphor screens (Kodak, now Carestream Health, New Haven, CT) for 48 hr and hybridization signal was read using a Storm System phosphor imager (Molecular Dynamics, Amersham Biosciences, Piscataway, NJ). Total hybridization signal

was quantified using ImageQuant version 1.2v as per Semsar and Godwin (2003), by defining regions of constant size across brains on all sections showing signal, summing to arrive at measurements for the preoptic area, optic tectum, and ventral telencephalon, and dividing by standard length (mm) for each animal.

Data analysis: We performed statistical comparisons using JMP 6.0.0 (SAS Institute, Cary, NC). The total hybridization signals for aromatase in the preoptic area of the hypothalamus were compared across phenotypic groups using analysis of variance (ANOVA).

Results

Cloning of partial aromatase cDNA

We cloned a portion of the cDNA from the cyp19 gene that encodes aromatase in brain tissue in fishes, commonly termed P450aromB or CYP19A2 (Blazquez and Piferrer, 2004). The 416bp sequence is shown with its translated amino acid sequence in Figure 1. An alignment of bluehead brain aromatase with other teleost brain aromatases is shown in Figure 2. A BLAST (National Center for Biotechnical Information) search using the bluehead brain aromatase nucleotide sequence indicated 91% sequence identity to *Pseudolabrus japonicus* brain aromatase (DQ298136), 89% sequence identity to *Halichoeres tenuispinis* brain aromatase (AY489060), 84% sequence identity to *Dicentrarchus labrax* brain aromatase (AY138522), 82% sequence identity to *Tilapia mossambica* brain aromatase (AF135850), 82% sequence identity to *Fundulus heteroclitus* brain aromatase (AY428666), 83% sequence identity to *Mugil cephalus* brain aromatase (AY859423), and 81% sequence identity to *Oreochromis niloticus* brain aromatase (AF306786).

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        10        20        30        40        50        60        70
GCCCACTACACCGCCAGATTTGGGAGCAGAACAGGGCTGGAGTGTATCGGGATGGAAGGGAAGGGAATCAT
 A  H  Y  T  A  R  F  G  S  R  T  G  L  E  C  I  G  M  E  G  K  G  I  I

        80        90       100       110       120       130       140
CTGCAACAGTGATGTCCACCTTTGGAAGAAAGCGAGGACATATTTCTCCAAAGCTCTGACAGGCCCTGCCT
  C  N  S  D  V  H  L  W  K  K  A  R  T  Y  F  S  K  A  L  T  G  P  A

       150       160       170       180       190       200       210
CCAGAGGACGGTGGGAATCTGTGTGAGCACCACAGCCAAACATCTGGATCGCCTACAAGAGATGACGGATT
S  R  G  R  W  E  S  V  *  A  P  Q  P  N  I  W  I  A  Y  K  R  *  R  I

      220       230       240       250       260       270       280
CATCTGGACATGTGGACGCCCTGAATCTGCTGAGAGCCACCGAGGTGGACATCTCCAACAAGCTTTTCCTC
 H  L  D  M  W  T  P  *  I  C  *  E  P  P  R  W  T  S  P  T  S  F  S  S

     290       300       310       320       330       340       350
AGAGTGCCTATTAATGAAAAAGACCTGCTGGTGAAAATCCATCACTACTTTGAAACATGGCAGACGGTTCT
  E  C  L  L  M  K  K  T  C  W  *  K  S  I  T  T  L  K  H  G  R  R  F

   360       370       380       390       400       410
AATAAAGCCTGATATATTCTTTAAGATTGGGTGGCTCTACAACAAGCACAAGAAAGCAGCA
*  *  S  L  I  Y  S  L  R  L  G  G  S  T  T  S  T  R  K  Q  X
```

Figure 1. Nucleotide and deduced amino acid sequence of aromatase cDNA clone isolated from bluehead wrasse brain.



Figure 2. Alignment of nucleotide sequence of bluehead wrasse (*Thalassoma bifasciatum*) brain aromatase to known teleost brain aromatases.

As expected, the highest homologies were with other teleost brain aromatases, however, the BLAST search also revealed lower homologies to several ovarian aromatase isoforms, including, among others, 68% sequence identity to *Halichoeres tenuispinis* ovarian aromatase (AY489061), 67% sequence identity to *Pseudolabrus japonicus* ovarian aromatase (DQ298135), and 71% sequence identity to *Fundulus heteroclitus* ovarian aromatase (AY428665).

Aromatase in situ hybridization

Levels of aromatase mRNA hybridization signal were significantly different in the preoptic area of the hypothalamus between the three phenotypes (see Figure 3a, ANOVA, F_2=4.51, p=0.035). Abundance levels were highest in females versus both IP males and TP males (Tukey-Kramer post hoc tests, $p<0.05$). There was no significant difference in IP males versus TP males (Tukey-Kramer post hoc test, $p>0.05$). In the optic tectum, there was no difference in expression in IP males versus TP males (see Figure 3b, Tukey-Kramer post hoc test, $p>0.05$). Females, however, again showed significantly higher aromatase mRNA abundance than either IP males or TP males (Tukey-Kramer post hoc tests, $p<0.05$). An area of the ventral telencephalon was also measured for aromatase mRNA abundances (see Figure 3c). Females (n=5) showed higher levels in this area versus TP males (n=4, Tukey-Kramer post hoc test, $p<0.05$) while sample sizes were too low for IP males (n=2) to allow a comparison in this brain area.

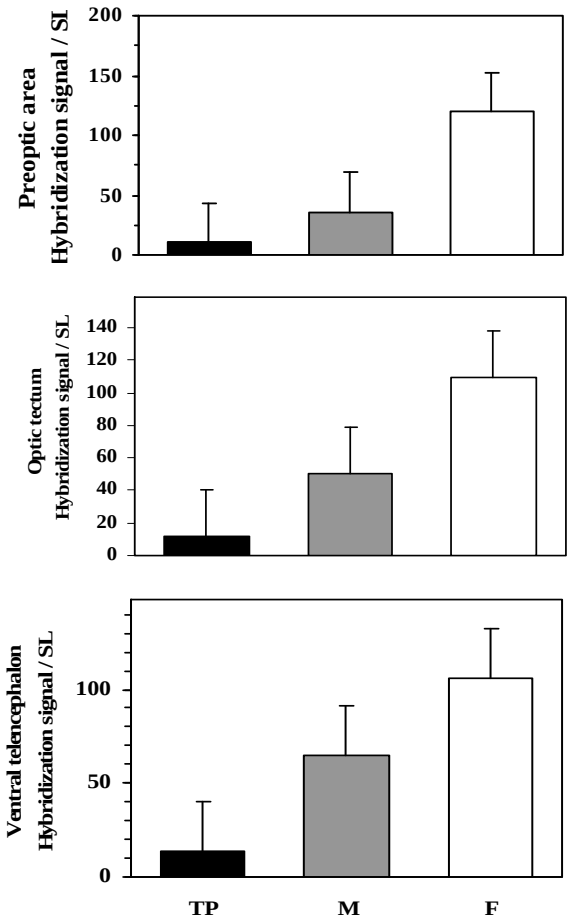


Figure 3. Aromatase mRNA hybridization signal (mean ± st dev; standardized by body size [SL]) for terminal phase (TP) males, initial phase males (M), and females (F) in the preoptic area of the hypothalamus, optic tectum, and ventral telencephalon.

Discussion

CYP19 genes have been found in mammals, birds, amphibians, teleosts, and cartilaginous fishes (see Choi et al., 2005). In most vertebrates, there is a single isoform of aromatase produced by one version of the CYP19 gene. Two isoforms of the CYP19 gene are expressed in teleosts, ovarian (CYP19A1) and brain (CYP19A2), and have been characterized in many fish species (among others: Callard et al., 1997; Forlano et al., 2001; Kishida et al., 2001; Valle et al., 2002; Blazquez and Piferrer, 2004; Choi et al., 2005). Interestingly, three isoforms of porcine CYP19 exist, ovarian, brain, and placental, and are encoded by three distinct genes (Graddy et al., 2000). We successfully cloned a portion of brain aromatase (CYP19A2) of the bluehead wrasse. The 416bp sequence showed higher homology with other teleost brain aromatases (81-91%) than to ovarian aromatases from other teleosts (67-71%). We then utilized this sequence to generate three antisense oligonucleotides for use in our *in situ* hybridization assay.

We hypothesized that, if estrogen is inhibiting the behavioral sex change process in the bluehead wrasse, aromatase mRNA expression would be highest in females versus IP males or TP males, and decrease as dominant status increased. We analyzed expression of brain aromatase mRNA and discovered significant differences in brain aromatase mRNA abundances among the three distinct sexual phenotypes of the bluehead wrasse. To our knowledge, this is one of a very few studies to compare brain aromatase mRNA abundances between behavioral phenotypes in specific brain nuclei (see also Forlano and Bass, 2005). We found that aromatase mRNA expression was higher overall in the POA of females than in either IP or TP males. This pattern of phenotypic variation in aromatase expression is in

agreement with other studies in our laboratory, including immunocytochemical assays suggesting greater staining intensity of aromatase-immunoreactive cells in the female and IP male brains, as well as cDNA microarray experiments in which the shift from subordinate to dominant in the hierarchy is correlated with decreases in aromatase expression (in preparation).

Evidence of inter- and intra-sexual variation in aromatase mRNA expression has also been shown in other teleosts. However, data regarding male-female differences across species lack consistency, as aromatase activity seems to be higher in females of some species and lower in females of other teleost species (reviewed in Forlano et al., 2006). In zebrafish (*Danio rerio*), both sexes exhibit clear intrasexual variation in the neural aromatase phenotype. 'High-expressing' zebrafish males and females were found to have higher aromatase mRNA in the telencephalon and hypothalamus when compared with 'low-expressing' zebrafish (Goto-Kazeto et al., 2004). In the midshipman (*Porichthys notatus*), aromatase mRNA levels vary seasonally, with females having the highest levels in the POA during gonadal recrudescence and Type I males having higher levels at the start of the courtship period (Forlano and Bass, 2005). Brain homogenates of male pejerrey (*Odontesthes bonariensis*) showed similar variation between the sexes, with males having higher aromatase mRNA expression in the forebrain and midbrain (Strobl-Mazzulla et al., 2005). Another study using whole brain homogenates of juvenile sea bass (*Dicentrarchus labrax*) reports a more complex pattern, with higher aromatase mRNA levels in females than in males, switching after the period of gonadal sexual differentiation to lower levels in females (Blazquez and Piferrer, 2004). In some cases, these inconsistencies in male-female

differences in aromatase expression/activity might arise from seasonal changes in circulating steroid levels or reproductive state (Pasmanik et al., 1988a, b; Forlano and Bass, 2005). In the plainfin midshipman, however, even non-reproductive females maintained elevated levels of aromatase mRNA expression in the POA (Forlano and Bass, 2005).

Aromatase mRNA and protein levels are not always tightly correlated, since transcription is not the only level at which aromatase activity is regulated (Balthazart et al., 2006, Forlano, Schlinger and Bass, 2006). Given that aromatase activity can be regulated at various levels, there are both advantages and limitations in using mRNA quantification as opposed to functional protein and/or aromatase activity assays. This issue has been particularly well studied in the Japanese quail, where many studies strongly link increases in aromatase mRNA expression to increases in actual enzymatic activity (Panzica, Viglietti-Panzica and Balthazart, 1996, Balthazart, Baillien and Ball, 2001, Balthazart et al., 2006, Cornil, Ball and Balthazart, 2006). In quail, independent studies using a measure of enzymatic activity, semi-quantitative evaluation of the aromatase protein based on the number of aromatase-immunoreactive cells, and quantification of aromatase mRNA by RT-PCR all indicate that testosterone markedly increases the levels of the aromatase enzyme (Balthazart, Baillien and Ball, 2001). However, it is the marked increase in the magnitude of testosterone's effect on enzymatic levels that points to multiple mechanisms of control (Balthazart et al., 2003, Balthazart, Baillien and Ball, 2001). There are many reasons for poor correlations between aromatase mRNA expression and enzymatic activity. These include, but are not limited to, varied post-transcriptional mechanisms, pre- and post-translational interactions, substantial variation in the half life of the protein, substrate availability (i.e.

steroid synthesis and degradation), location and abundance of steroid receptors, and modulation of steroid receptors by various cofactors (Ball and Balthazart, 2006, Balthazart and Ball, 1998, Balthazart et al., 2003, Charlier and Balthazart, 2005, Charlier et al., 2006, Panzica, Viglietti-Panzica and Balthazart, 1996, Rupprecht and Holsboer, 1999). Recognizing these limits on interpretation, mRNA abundances remain an informative and useful measure for several reasons. These include the high spatial resolution offered by *in situ* hybridization and relatively direct quantification possible with the use of radiolabeled probes. These features are useful in assessing possible sex differences as molecular and cellular changes are not always reflected over an entire brain region (Panzica, Viglietti-Panzica and Balthazart, 1996). Also, aromatase mRNA levels are not subject to the same degree of rapid alteration as aromatase activity, which can be affected over very short time scales by dopaminergic inputs and changes in intracellular calcium concentrations (e.g. Balthazart et al., 2003). We have shown tyrosine hydroxylase-immunoreactive fibers in close proximity to aromatase-ir cells in the bluehead preoptic area, suggesting dopaminergic effects on aromatase activity could also be important, but have not further explored possible dopamine effects in this system. In our experiments, fish are sacrificed on the boat within two minutes of capture. Although this sampling is fast, given the evidence above, a few minutes is conceivably enough time for an effect on aromatase activity to occur. Measures of aromatase mRNA expression, however, should remain unaltered over this time scale. The enzymatic activity of aromatase is obviously involved in rapid (within minutes) control of behavior, but aromatase mRNA might better reflect the maintenance of these rapid behavioral adaptations required to assume territorial status.

What might be the functional significance of sex differences in brain aromatase mRNA expression? Forlano et al. (2006) lists five potential roles for locally produced neural estrogen, including the potential to modulate transcription through nuclear or even membrane estrogen receptors and activating signal transduction pathways for rapid modulation of cellular physiology. In attempting to understand sex differences in aromatase expression in certain brain nuclei, the bluehead wrasse is an excellent model system because the behavioral phenotypes are distinct, both developmentally and in terms of behavioral profile. The behavioral changes associated with a shift in social dominance are very pronounced, occur rapidly, and can be manipulated in the full complexity of the natural environment.

In the bluehead wrasse, as in other sex-changing fishes, an unpredictable social system requires rapid adaptation to changing dominance hierarchies. Blocking aromatase activity with specific inhibitors can induce gonadal sex change under inhibitory social conditions in many teleost species (Bhandari et al., 2004; Kroon and Liley 2000; Kroon et al., 2005), including the bluehead wrasse (Austin et al., unpublished). These studies suggest that estrogens are central players in at least the gonadal components of sex change, but it is unlikely that these gonadal changes possess the temporal specificity to regulate behavioral changes that occur with rapid and dramatic changes in social status. For example, as mentioned earlier, in the bluehead wrasse male-typical behavior is often exhibited by sex changing females within minutes of removal of the TP male, but gonadal change takes place over 8-10 days (Godwin, Crews and Warner, 1996, Robertson,1972, Warner and Swearer, 1991). Patterns in other species suggest that alterations in neural aromatization are potential mechanisms underlying fast-acting behavioral adaptations. The plainfin midshipman

(*Porichthys notatus*) exhibits two distinct male phenotypes that show pronounced differences in behavior. These behavioral differences are correlated with differences in both aromatase activity and mRNA expression in various brain regions involved in sexual and aggressive behavior (Schlinger et al., 1999; Forlano et al., 2001; Forlano and Bass, 2005; Forlano et al., 2005). In the blue-banded goby (*Lythrypnus dalli*), there are similar sex differences, with females having significantly higher neural aromatase activity than males. A rapid decrease in aromatase activity is seen at the onset of socially-induced sex-change and associated increases in aggressive behavior (Black et al., 2005a, b).

We have provided evidence of differences in aromatase expression between the three distinct sexual phenotypes of the bluehead wrasse that are consistent with estrogenic inhibition of male-typical courtship and aggression. Our findings suggest that neural estrogen synthesis could mediate rapid behavioral adaptation, although this has not been directly tested here, and could play a role in maintenance of behavioral differences between phenotypes. The next step is to experimentally test this in the field, utilizing systemic estrogen implants to mimic changes in estrogen synthesis in various social situations. Intracranial implantation of estrogen and aromatase inhibitors would help to pinpoint the response in key brain regions. Also, measures of aromatase activity over the course of behavioral sex change would aid in characterizing the nature and time course of the relationship between aromatase and any behavioral shifts. It may be that these local changes in estrogen synthesis are part of a feedback loop regulating neural estrogen and culminating in the assumption of male-typical dominant, aggressive behavior.

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CHAPTER IV

ESTRADIOL INHIBITS SOCIALLY CONTROLLED SEX CHANGE IN THE BLUEHEAD WRASSE, THALASSOMA BIFASCIATUM

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Estradiol inhibits socially-controlled behavioral sex change in the bluehead wrasse, *Thalassoma bifasciatum***.**

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SUMMARY

Estrogens activate male-typical sexual behavior in several mammalian and avian models. Estrogen signaling also appears critical in the control of sex change in some fishes, where decreases in estradiol levels permit development of male behavior. The bluehead wrasse is a protogynous hermaphrodite that exhibits rapid increases in aggressive and male-typical courtship behavior as females undergo sex change. Removal of the ovaries does not prevent these changes. In field experiments involving gonadally-intact and gonadectomized females, estradiol implants prevented behavioral sex change in large females with a high likelihood of becoming socially dominant after removal of the dominant males. By contrast, cholesterol-implanted control females showed full behavioral sex change, along with a higher frequency of aggressive interactions and male-typical courtship displays than E_2-implanted animals. Following the field studies, we used *in situ* hybridization to assess aromatase mRNA levels. Aromatase mRNA expression was higher in the POA of E_2-implanted gonadally-intact females than cholesterol-implanted controls. The correlation between increased mRNA abundances and non-dominant behavioral roles suggests that neurally-produced estradiol could regulate behavioral sex change. It is possible that this regulation occurs through estrogen's effects on other neural signaling systems that underlie reproductive behavior.

Key words: aromatase, estradiol, social behavior, aggression, preoptic area

Short title for page headings: Estrogenic inhibition of behavioral sex change

1. INTRODUCTION

Estrogens have been implicated in the control of a wide range of sexual and social behaviors across vertebrates. In well-studied mammalian and avian models, an increase in neurally synthesized estrogen activates male sexual behavior (Resko *et al.* 2000; Bakker *et al.* 2004; Balthazart *et al.* 1996). Several studies have also highlighted the importance of estrogens in regulating sex change in fishes (Devlin & Nagahama 2002; Godwin *et al.* 2003; Frisch 2004). In contrast to findings in mammals and birds, recent work suggests it is actually a decrease in E_2 levels that permits male-typical sexual behaviors to develop during sex change (Kroon *et al.* 2005; Black *et al.* 2005). Behavioral sex change can begin within minutes to hours of the appropriate stimulus in sex changing fishes (Robertson, 1972; Warner & Swearer 1991), and rapid neural changes have also been documented (Black *et al.* 2005). The hormonal effects of estrogens are typically thought to work relatively slowly through ligand-activated transcription factors. While these classical pathways are clearly important, increasing evidence also points to a role for rapid steroid actions (Remage-Healey & Bass 2004, 2006a, 2006b; Revankar *et al.* 2005). Social influences acting on the brain may require a fast-response pathway, and production of neurosteroids can be rapidly altered in the brain. Estrogen is produced in the brain by aromatization of testosterone. Changes in aromatase activity in the quail brain can be seen within minutes of pharmacological manipulation and are reversible, suggesting physiological events could potentially regulate estrogen production in the brain over short time courses (Balthazart *et al.* 2001).

Sex changing fishes provide dramatic examples of rapid responses to social interactions. Accumulating evidence indicates a key role for estrogen signaling as an underlying

mechanism during female-to-male sex change. In experiments with hermaphroditic fish, aromatase inhibitors induced complete protogynous (female-to-male) sex change in the protogynous blackeye goby, *Coryphopterus nicholsii* (Kroon & Liley 2000), and blocked natural sex change in the protandrous (male-to-female) black porgy, *Acanthopagrus schegeli* (Lee *et al.* 2001). In a protogynous wrasse, *Halichoeres trimaculatus*, administration of an aromatase inhibitor concurrently with an androgen induced sex change, while exogenous estrogen treatments suppressed the induction of sex change (Higa *et al.* 2003). The neural form of aromatase is abundantly expressed in the brains of teleost fishes (Callard *et al.* 2001), including in key regions regulating sexual behavior (Schlinger *et al.*, 1999; Forlano *et al.* 2001; Forlano & Bass 2005a, b; Chang *et al.* 2005; Kishida & Callard 2001; Menuet *et al.* 2003, 2005). Neural aromatase activity has been shown to decrease significantly within hours after male removal in *Lythrypnus dalli*, a gobiid species with socially controlled sex change (Black *et al.* 2005). This decrease in aromatase activity is correlated with rapid increases in aggressive and territorial behavior in females undergoing protogynous sex change.

We have been investigating the role of estrogens and aromatase in the sex change process of the bluehead wrasse, a protogynous hermaphrodite with socially controlled sex change. Sex-changing female wrasses initiate male sexual and aggressive displays within minutes of removal of the dominant male, while color and gonadal changes occur more slowly and are complete within 8-10 days (Warner & Swearer 1991). The entire sex change process will occur in socially permissive environments even when the experimental female has been gonadectomized (Godwin *et al.* 1996). This highlights the importance of

understanding neural signaling mechanisms in the bluehead wrasse's behavioral control pathway. In immunocytochemical studies in our lab, we also found aromatase-immunoreactive cells in areas that are critical in regulating male-typical sexual behavior including, among other brain areas, the dorsal and ventral telencephalon, the preoptic area of the hypothalamus, and the lateral recess of the third ventricle (Marsh *et al.* 2006). In other experiments, we found that when the aromatase inhibitor 1,4,6-androstatrien-3,17-dione (ATD) is given alone or coadministered with testosterone, complete color and gonadal sex change is induced in female bluehead wrasses (Austin *et al.* unpublished). Rapid alterations of sexual behavior in response to changing social conditions in the bluehead wrasse could be due to changes in neural estrogen synthesis.

To directly assess the influence of estrogens on behavioral sex change process of the bluehead wrasse, we tested whether exogenous estradiol could block behavioral sex change in gonadally-intact or gonadectomized female bluehead wrasses under field conditions. We also measured aromatase mRNA expression in the preoptic area of the hypothalamus to look for estradiol effects on aromatase expression.

2. MATERIALS AND METHODS

***(a) Study site and species*:** All experiments were conducted on patch reefs located near Glover's Reef Marine Research Station (Middle Caye, Glover's Reef, Belize) during April to June 2006 or January to February 2007. All initial phase (IP) bluehead wrasses (male and female) that were > 40mm standard length (SL) on small to medium sized patch reefs (2-4 spawning sites) were captured by lift-net between 0730h-1030h each day. Following

capture, fish were held at the field laboratory until ~1500h then measured (standard length, SL, in mm) and sexed by external genital papilla observation. Individual experimental females were marked with Floy tags and different color combinations of plastic seed beads for recognition of individual fish.

***(b) Experimental methods*:** Surgeries were performed as in previous studies (Godwin *et al.* 1996; Semsar & Godwin 2003, 2004) and involved the insertion of slow-release Silastic tubing implants used to manipulate estradiol levels in either gonadally intact (experiment 1) or ovariectomized females (experiment 2). Silastic implants (Silastic tubing, 1.47 mm ID, 1.96 mm OD, Dow Corning, Midland, MI; 10-11mm length) were packed with either crystalline estradiol (E_2; 8mm packed length) or cholesterol (CHL; 8mm packed length) and placed in the abdominal cavity. Fish recover quickly from surgery and are returned to their home reef the same day (survival rate 90-100%), active and feeding the following day, and grow new scales over the surgical wound within a week. All smaller females were also returned to their home reef, while IP males were relocated to similar reefs nearby. All experimental methods were approved by and are in compliance with the guidelines of the Institutional Animal Care and Use Committee of North Carolina State University.

Dominant terminal-phase males were removed from the reef after females had to and the first spawning period they experienced as dominants was considered 'Day 1,' meaning the first day of social conditions permissive to sex change on the reef. In the gonadally-intact experiment, Experiment 1, fish were observed on Days 1, 3, and 7 during April of 2006. Treatment reefs (n=4) included those on which the largest females were implanted with a

Silastic implant containing E_2 while smaller females were implanted with cholesterol (CHL). On control reefs (n=3), this was reversed, with the largest females receiving CHL implants. In Experiment 1, with intact animals, mean body size overall was 59.86 ± 4.5mm SL; 58.8 ± 3.6mm SL for E_2 implanted fish and 61.0 ± 5.4mm SL for CHL implanted fish. In Experiment 2, females were ovariectomized and only observed on Day 7 following TP male removal. On these reefs (n=17 total), fish were implanted with alternating treatments in descending size order, with the starting treatment chosen randomly, with 4 reefs used during May to June of 2006 and the remaining 13 reefs used in January-February of 2007. In Experiment 2, with ovariectomized animals, mean body size overall was 62.2 ± 5.6 mm SL; 67.3 ± 5.7mm SL for E_2 implanted fish and 61.2 ± 2.4mm SL for CHL implanted fish. Standard length in E_2 and CHL-implanted animals did not significantly differ between the two experiments (p=0.601).

Observations were made during the daily spawning period, which averages approximately 150 minutes and occurs between 1030h-1500h. An observer quantified male behaviors during ten minute focal individual observations, including the frequency and duration of aggression towards conspecifics, feeding bites, and courtship behavior, including rapid fin movements, spawns, 'loops', and inspections (see Semsar *et al.* 2001 for detailed descriptions). At the end of each experiment, fish are sacrificed immediately on recapture (within approximately three minutes on the boat) using an overdose of MS-222 (tricaine methanesulfonate) and their brains dissected out and fixed in 4% paraformaldehyde. After 24 hours fixation, brains were placed in 0.1M phosphate buffer, and held at 4° C until shipment to our Raleigh laboratory for processing.

***(c) Cloning of bluehead aromatase cDNA*:** We partially cloned the brain isoform of the *cyp19* gene that encodes aromatase, commonly termed P450aromB or CYP19A2 (Blazquez & Piferrer 2004). RNA was isolated from field-collected fish then reverse transcribed to cDNA using the OmniScript® RT kit (Qiagen, Valencia, CA) and Oligo(dT)15 primers (Promega, Madison, WI). The resulting cDNA was used as template in two polymerase chain reaction (PCR) amplifications of P450aromB. Two sets of nested degenerate oligonucleotide primers were designed based on an alignment of sequences of CYP19A2 genes from teleosts [*Carassius auratus* (GenBank accession number AB009335), *Danio rerio* (120031), *Oreochromis niloticus* (AF306786), *Porichthys notatus* (AF472578), and *Tilapia mossambica* (AF135850)]. In the first amplification, forward primer (5`-TCCKYYCTTCTTRGCAGG-3`) and reverse primer (5`-CCAGGATGGCCTTCATCATCA-3`) were predicted to amplify a 1211bp product from the consensus sequence. In the second amplification, nested primers (5`-GCCCACTACACMKCCAGATTT-3` and 5`-GCTGCTYTCTTRTGCTTG-3') were used to reduce background and amplify a 416bp product. This PCR product was cloned into pCRII (Invitrogen, Carlsbad, CA), transformed, amplified, purified and sequenced to confirm the identity of the clone as CYP19A2 using BLAST (NCBI). Two clones contained a match for other teleost aromatase CYP19A2 sequences. Recent alignments show a high degree of similarity between our partial brain aromatase sequence and those of other wrasses; sequences which were not available at the start of our project (*Pseudolabrus japonicus* (DQ298136.1), E value 5e-163 and *Halichoeres tenuispinis* (AY489060.1), E value 6e-156).

***(d) In situ hybridization*:** Hybridization signal was used to assess the relative abundance of brain aromatase as described previously for assessing mRNA abundances in bluehead wrasse brain tissue (Godwin *et al.* 2000; Semsar & Godwin 2003) with some differences as described below. Brains were cryoprotected in 30% sucrose for 24h prior to sectioning into six adjacent series of 20 μm sections and held at -80°C until processed in assays. Three specific, non-degenerate, non-overlapping antisense oligonucleotide probes were designed against the 416 bp partial clone sequence of bluehead brain aromatase (5`-AAATAATCCCCTTCCCTTCCATCCCGATGCACTCCAGCCC-3`; 5`-CCACATGTCCAGAAGGATCGGTCATCTCTTTTAGGCGCTC-3`; and 5`-ATTAATCGGCACCCTGAGGAACAGCTTATTGGAGATGTCC) A mixture of equal amounts of these three oligonucleotides was end-labeled with ^{33}P-dATP (specific activity, 3000 Ci/mmol; Perkin Elmer, Boston, MA), added to hybridization buffer, and applied to slides as described previously (see Godwin *et al.* 2000; probes were added at 2 X 10^6 DPM/oligonucleotide probe for 6 X 10^6 DPM/slide). Following washes, dry slides were placed against GP phosphor screens (Kodak, Toronto) for 48 hr and hybridization signal was read using a Storm System phosphor imager (Molecular Dynamics, Amersham Biosciences, Piscataway, NJ). Total hybridization signal was quantified using ImageQuant version 1.2v as per Semsar & Godwin (2003), by defining regions of constant size across brains on all sections showing signal, summing to arrive at measurements for the preoptic area, and dividing by standard length (mm) for each animal.

(e) Data analysis: Statistics were performed using JMP 6.0.0 (SAS Institute, Cary, NC).

Behavioral scores were averaged over three 10min observation periods. Behavioral experiments were analyzed using oneway analysis of variance (ANOVA) to compare frequencies and durations of behaviors, including courtship displays and male-typical inspections, and repeated measure multiple analyses of variance (MANOVAs) to compare these behaviors across multiple days where applicable. The total hybridization signals for aromatase in the preoptic area of the hypothalamus were compared across treatment groups using analysis of variance (ANOVA).

3. RESULTS

***(a) Effects of estrogen implants in gonadally-intact females under socially permissive conditions*:** Behavioral sex change was prevented in females that received E_2 implants (n=10), but not in the sham-implanted animals (n=8). The frequency of aggressive interactions on Days 1, 3, and 7 was significantly lower in E_2-implanted animals (MANOVA, F=15.76, p=0.001), as was the frequency of male-typical courtship displays (MANOVA, F=6.56, p=0.023; Figure 1). Neither the mean duration of aggressive interactions nor the mean duration of courtship displays differed significantly between treatment groups (MANOVA, F=2.31, p=0.151 and F=2.04, p=0.175, respectively). Other measures of male-typical courtship behaviors, loops and inspections, did not show significant differences between E_2-implanted and CHL-implanted animals (MANOVA, F=3.07, p=0.102 and F=1.23, p=0.283, respectively). While feeding bites on the substrate, a measure not related to sexual motivation, were not significantly different between the two treatments overall (MANOVA, F=0.04, p=0.852), there were significantly more feeding bites by E_2-implanted

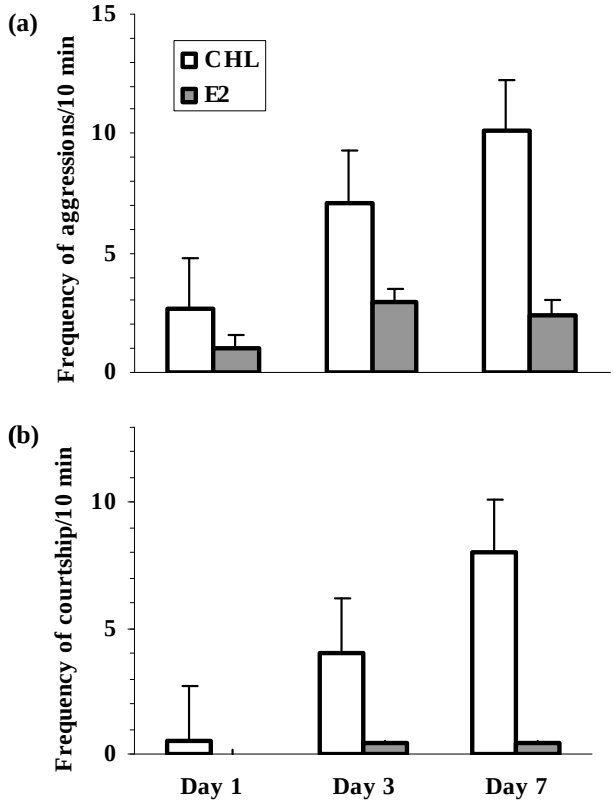


FIGURE 1. Frequency of aggressive interactions and courtship behaviors over three averaged 10 min observation periods (mean ± SEM) for Days 1, 3 and 7 in gonadally-intact CHL-implanted animals versus E_2-implanted animals, respectively.

fish on Day 3 than by CHL-implanted animals (oneway ANOVA, F=10.169, p=0.005). This difference was reversed on Day 7, with CHL-implanted animals showing significantly more feeding bites than E_2-implanted fish (oneway ANOVA, F=23.834, p=0.0002).

***(b) Effects of estrogen implants in ovariectomized females under socially permissive conditions*:** There was no difference in behavioral scores between study years for the experiments using ovariectomized females (MANOVA, F=0.147, p=0.703). Data were therefore combined for analysis. Behavioral sex change was prevented in ovariectomized (OVX) females that received E_2 implants (n=16) but not in OVX females that received cholesterol (CHL) implants (n=16). The frequency of aggressive interactions was significantly lower in E_2-implanted animals (F=6.46, p=0.039; Figure 2), with no difference in the duration of aggressions (F=5.11, p=0.078; Figure 2). Both the frequency and duration of male-typical courtship displays were significantly lower in E_2-implanted animals relative to controls (F=6.14, p=0.005 and F=5.19, p=0.009 respectively; figure 2). Neither the male-typical courtship loops nor feeding bites on the substratum were different between the two treatments (F=2.43, p=0.993 and F=1.26, p=0.294, respectively). However, male-typical inspection behaviors were significantly higher in OVX animals treated with CHL (F=4.33, p=0.019).

***(c) Aromatase expression in the preoptic area of the hypothalamus*:** Levels of aromatase mRNA hybridization signal were significantly higher in the preoptic area of gonadally-intact E_2-implanted females than cholesterol-implanted controls (n=7 and 6, respectively, ANOVA,

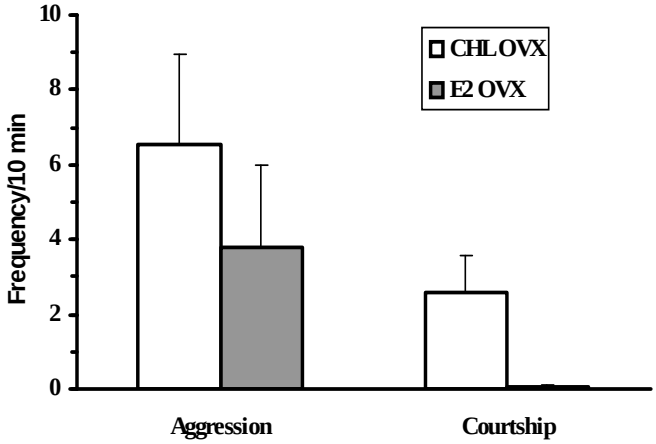


FIGURE 2. Frequency of aggressive interactions and courtship behaviors over three averaged 10 min observation periods (mean ± SEM) for Day 7 in ovariectomized CHL-implanted animals versus E_2-implanted animals.

F=10.70, p=0.007; Figure 3). As an internal control, an area of the optic tectum was measured for hybridization signal across the animals, and showed no difference in E_2-implanted females versus cholesterol-implanted controls (ANOVA, F=0.013, p=0.912). In ovariectomized animals, levels of aromatase mRNA hybridization signal were not significantly different in the preoptic area of E_2-implanted females than cholesterol-implanted controls (n=5 and 3, respectively, ANOVA, p=0.930, F=0.008). In ovariectomized animals, the optic tectum area measured again showed no difference in hybridization signal between OVX females receiving E_2 versus CHL (ANOVA, p=0.126, F=3.15).

4. DISCUSSION

The bluehead wrasse offers a behavioral system in which the social environment plays a pivotal role in the development of a male phenotype. We provide evidence here that estradiol inhibits the development of male-typical sexual behaviors in female wrasses, even under social conditions where behavioral sex change by those females would be expected. In our experiments, the inhibition of the development of male behavior was coupled with greater expression of aromatase mRNA in the preoptic area of the hypothalamus, a brain region critical to the regulation of reproductive behavior.

In the sex change process of the bluehead wrasse, gonadal change and behavioral change are decoupled. This decoupling occurs both temporally, because behavioral sex change greatly precedes gonadal change (Warner & Swearer 1991), and physiologically, because ovariectomy neither stimulates nor prevents development of male-typical behavior when a female becomes socially dominant (Godwin *et al.* 1996). Instead it appears that

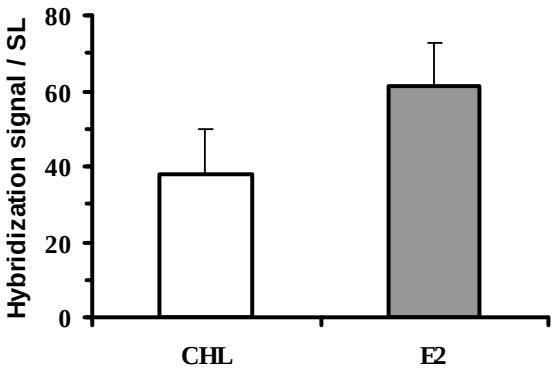


FIGURE 3. Aromatase mRNA hybridization signal (mean ± SEM; standardized by body size [SL]).

neurally produced estrogen plays an important role in the behavioral sex change process of the bluehead wrasse. In this system, an estrogen suppression mechanism capable of being "turned off" rapidly (i.e., through inhibition of aromatase activity and/or reductions in aromatase protein levels) could allow rapid behavioral adaptation to changing social circumstances. Rapid changes in aromatase activity are seen with socially-mediated sex change in another fish, the bluebanded goby, *Lythrypnus dalli*, where male removal induces rapid decreases in neural aromatase activity in females as they undergo sex change (Black *et al.* 2006).

There is evidence from other fishes that E_2 can block gonadal sex change and that the lack of E_2 can stimulate it (Kroon *et al.* 2000, 2003, 2005). However, the present findings are the first evidence that we are aware of that maintaining high levels of E_2 and aromatase expression, as indicated by increased aromatase mRNA abundances, can also block the behavioral aspects of this transition. In our field experiments, exogenous E_2 prevented females from exhibiting dominant male-typical sexual and territorial behavior under social conditions that normally promote protogynous sex change in this species (Warner & Swearer 1991; Godwin *et al.* 1996; Semsar & Godwin 2003, 2004). This was true regardless of whether these females were gonadally-intact or ovariectomized. These findings are consistent with the prediction that endogenous estrogen suppresses behavioral sex change in a socially-inhibitory environment. This endogenous estrogen could be either produced peripherally by the gonads, or locally synthesized in the brain. In the gonadally-intact females, the effects of exogenous estrogen could have been due to actions in either the brain or gonads (e.g., estrogen stimulating the production of some other behaviorally active hormone from the

ovaries). Experiments using ovariectomized females, however, provide evidence that estrogenic suppression of sexual behavior does not depend on other products of the gonads. A second issue concerns the site of action of exogenous estrogen in blocking behavioral sex change. Pinpointing critical brain regions where estrogenic inhibition might be mediated would likely require intracranial implantation of estradiol (Barfield & Chen 1977; Pleim *et al*. 1988; Nyby *et al*. 1992).

What brain region or regions are likely to be the key sites of estrogen production and its inhibitory actions? In a number of vertebrates, the highest levels of neural aromatase activity have been localized to parts of the limbic system, especially hypothalamic areas (Roselli *et al.* 1985; Schumacher *et al.* 1987; Schlinger *et al.* 1999; Naftolin *et al.* 2001), brain regions critical in the mediation of sociosexual behaviors. Localization of aromatase expression in the mammalian brain also has focused on the hypothalamus, since it is the 'classical target' of estrogen action (Beyer 1999), and represents a critical integrator for neuroendocrine actions. Expression of aromatase in key brain regions regulating sexual behavior also holds true in fishes (Schlinger *et al*. 1999; Forlano *et al*. 2001; Forlano & Bass 2005a, b; Chang *et al*. 2005; Kishida & Callard 2001; Menuet *et al*. 2003, 2005). Likewise, we have found similar results using immunohistochemistry, with aromatase-immunoreactivity seen in the dorsal and ventral telencephalon, the preoptic area of the hypothalamus, and the lateral recess of the third ventricle, among other brain areas (Marsh *et al.* 2006). It is important to note that fishes, however, have two different aromatase genes which encode two structurally different proteins that are then preferentially expressed in either the gonad or the brain (reviewed in Piferrer & Blazquez 2005). In the brains of female

bluehead wrasses from our behavioral field experiments, the prevention of behavioral sex change by E_2 implants was correlated with greater abundances of brain aromatase mRNA in the preoptic area of the hypothalamus.

Differences in aromatase expression have been implicated in sexual differentiation and maturation in many species, often in the induction of male versus female developmental pathways (Schlinger *et al.* 1999). The plainfin midshipman, *Porichthys notatus*, is a fish that exhibits two male phenotypes with distinct behavioral profiles. Plasma steroid hormone levels vary across reproductive season with peaks of T and 11-ketotestosterone (11KT) occurring in dominant (Type I) males. Females instead show increases in both E_2 and T, with both females and subordinate (Type II) males having greater overall levels of aromatizable T than males (Sisneros *et al.* 2004). These changes in circulating sex steroid levels are correlated with sex differences in reproductive behavior, and with differences in brain aromatase activity (Schlinger *et al.* 1999). These authors suggest that the local conversion of T by aromatization prevents masculinization of the sonic motor nucleus, a structure critical in control of vocalizations used in male sexual behavior, thereby helping direct the development of separate male phenotypes. (Schlinger *et al.* 1999; Forlano *et al.* 2001). The model (see figure 4) we propose for the sex change process in the bluehead wrasse predicts that neural changes leading to male-typical sexual and aggressive behavior are inhibited by estrogen derived through local aromatization.

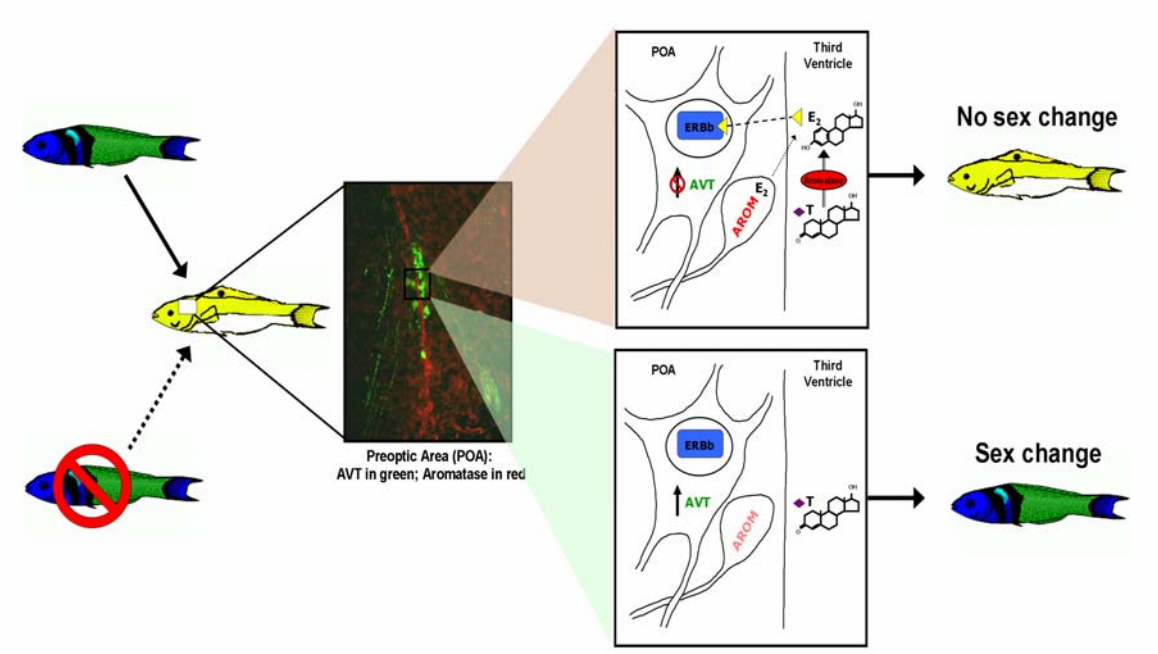


FIGURE 4. Proposed model for estrogenic suppression of behavioral sex change. In a socially inhibitory environment (i.e. TP male present), subordinate fish experience higher levels of estradiol due to aromatization. Neural estrogen levels are increased, which then act to suppress the increase in AVT expression that is seen in socially dominant fish.

Classically, studies on sexual differentiation and the development of male sexual behavior have focused on T as the main determinant. Our experiments emphasize the importance of estrogen in the development of sexually dimorphic phenotypes in the bluehead wrasse. A recent study in mice demonstrated that 'knocking out' neural inputs of the vomeronasal organ (VNO) induced robust male-like sexual and courtship behaviors in female mice (Kimchi et al. 2007). The authors suggest dual circuits controlling male and female-typical behaviors respectively coexist in the female mouse brain and that their activity is controlled by hormonal and environmental modulators (Kimchi et al. 2007). Similarly, in the bluehead wrasse and other sex-changing fishes where behavioral transitions can occur in minutes to hours, it appears likely that a functional male behavioral circuit is present prior to sex change, one that consists of both male and female sexual behavior under the control of different endogenous and exogenous inputs. Estrogenic inhibition could then be considered a potential modulator of behavior, in this case suppressing male-typical sexual behavior. Decreased inhibition, due to changing socially-derived sensory cues, could stimulate the male-typical behavior to be unmasked. We propose that a decrease in estrogenic inhibition is responsible for the regulation of male-typical sexual behavior in the bluehead wrasse. This is consistent with another proposal of aromatase activity driving behavioral sex change, based on data from a sex-changing fish showing a rapid decrease in brain aromatase activity correlated with an increase in aggressiveness (Grober 1997; Black *et al*. 2005). It is also consistent with rapid modulation of aromatase activity and rapid estrogenic effects seen in other vertebrate systems (Balthazart *et al*. 2001, 2002; Trainor *et al*. 2004; Remage-Healey & Bass 2006; Forlano *et al*. 2006). How might this estrogenic control of sexually dimorphic

behavior be regulated? What other gender-specific modulators might exist? What neurochemical cues might lie downstream of estrogen? Further exploration of these and other mechanisms occurring in varying social situations will aid in understanding sexual dimorphisms in behavior and, more generally, behavioral adaptation across taxa.

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CHAPTER V.

SOCIAL CONTROL OF BEHAVIOR:

POTENTIAL FOR ESTROGENIC SIGNALING PATHWAYS

The findings presented in the preceding chapters support our model of estrogenic inhibition of male-typical behavior and behavioral sex change in the bluehead wrasse. We have demonstrated the presence of aromatase-ir cells in brain nuclei important in neural control of behavior, and the potential for interactions with both vasotocinergic and dopaminergic pathways. We have also documented differences in aromatase mRNA expression across sexual phenotypes in a sex-changing fish. Additionally, we have shown that estradiol can block behavioral sex change under field conditions, and this blockage is associated with differences in aromatase mRNA abundances. These contributions are consistent with those of other studies indicated previously (see Chapters II – IV), and have helped to further our understanding of the role of neural estrogen in controlling behavioral adaptation. Although androgens were classically deferred to, it is now considered almost an established fact in the field of behavioral endocrinology that aromatization plays a critical function in the hormonal control of male sexual behavior (Ball and Balthazart 2006). However, aromatized estrogen in many species of fishes and birds is mainly thought to activate male behavior, not inhibit it (Adkins-Regan 1981, Brantley et al. 1993, Balthazart et al. 2004, Cornil et al. 06, Forlano et al. 2006). We propose that decreases in neural estrogen synthesis are responsible for the development and expression of male-typical sexual behavior in the bluehead wrasse. Estrogenic inhibition could then be considered a potential modulator

of behavior, in this case suppressing male-typical sexual behavior. Decreased inhibition, due to changing socially-derived sensory cues, could allow the male-typical behavior to be unmasked.

Potential Mechanisms of Estrogenic Inhibition

Regulation of the aromatase enzyme

How might the effects of aromatase on estrogen's role in sexually dimorphic behavior be regulated? As noted earlier in the chapter on phenotypic differences in aromatase expression, there are many factors that might regulate aromatase activity, thereby indirectly modulating behavior through estrogen availability. These include, but are not limited to, varied pre- and post-transcriptional, translational, and post-translational mechanisms including phosphorylation, substantial variation in the half life of the protein, substrate availability (i.e. steroid synthesis and degradation), and subcellular localization of aromatase (Ball and Balthazart, 2004, Balthazart and Ball, 1998, Balthazart et al., 2003, Baulieu,1998, Panzica, Viglietti-Panzica and Balthazart, 1996).

In many teleosts, as well as in birds and other vertebrates, estradiol can upregulate aromatase mRNA directly in a positive feedback manner (Forlano, Schlinger and Bass, 2006, Forlano, 2005, Gelinas, Pitoc and Callard, 1998, Kishida and Callard, 2001, Lee et al., 2000). Estrogen-response elements (EREs) were first found in the promoter regions of the goldfish and zebrafish CYP19B genes, suggesting estradiol could directly mediate aromatase transcription through one or more of the estrogen receptor subtypes (Callard et al., 2001, Kazeto et al., 2001, Tchoudakova et al., 2001). More recently, the presence of EREs has also

been confirmed in the promoter region of the aromatase genes of a variety of other teleosts (Chang et al., 2005, Kobayashi et al., 2005, Menuet et al., 2005, Nocillado et al., 2007, Pellegrini et al., 2005). Additionally, binding sites for steroidogenic factor 1 (SF1), sex determining region Y (SRY), Sry-related HMG box-9 (SOX 9), progesterone receptor, and androgen receptor have also been described in the teleost aromatase promoter region (see references in Forlano, Schlinger and Bass, 2006).

Regulation of estrogen

The modulation of estrogen itself, rather than only its availability, is also important. Molecular contributors that have more direct effects on estrogen's role in altering behavior include clearance or degradation of the locally produced estrogen, the cellular location and abundance of steroid receptors, and modulation of steroid receptors by various transcription cofactors (Ball and Balthazart, 2006, Balthazart et al., 2006, Bjornstrom and Sjoberg, 2005, Charlier and Balthazart, 2005, Charlier et al., 2006, Panzica, Viglietti-Panzica and Balthazart, 1996, Rupprecht and Holsboer, 1999). The question of localization of the targets of locally produced estrogen has received much attention. In contrast to other vertebrates, there are actually three ERs in teleosts, ERα, ERβb, and ERβa (Hawkins et al., 2000, 2005). The tissue distributions and binding affinities are distinct for each of these three ER subtypes in the Atlantic croaker (Hawkins and Thomas, 2004, Hawkins et al., 2005).

The relationships between these receptor subtypes and aromatase have been investigated in various species, including trout, plainfin midshipman and zebrafish, among other teleosts (Forlano, Deitcher and Bass, 2005, Menuet et al., 2003, Menuet et al., 2004, Pellegrini et al., 2005). Aromatase expression is high in the telencephalon, preoptic area and

mediobasal hypothalamus (Pellegrini et al., 2005), where both estrogen receptors and aromatase-expressing cells are known to be mainly expressed in trout (rtERα; (Anglade et al., 1994, Menuet et al., 2001, Menuet et al., 2003)), midshipman (Forlano et al., 2001, Forlano, Deitcher and Bass, 2005), and zebrafish (zfERα, zfERβ1, zfERβ2; (Menuet et al., 2002, Menuet et al., 2004). However, in teleosts, aromatase is expressed in glial cells, whereas ERs are found in neurons (Forlano et al., 2001, Goto-Kazeto et al., 2004, Menuet et al., 2003, Pellegrini et al., 2005). This lack of co-localization between aromatase and ERs highlights the importance in understanding steroid interactions with membrane receptors and pre- and post-synaptic targets (mini-review in Forlano, Schlinger and Bass, 2006). It is important to note, however, that co-localization of aromatase and estrogen receptor subtypes has only been addressed in two studies, and only involves one subtype, ERα (Menuet et al. 2003, Forlano et al. 2005). Data from a number of studies suggest that radial glial cells express estrogen receptors (reviewed in Pellegrini et al. 2005), and expression of estrogen receptor in glial cells has been documented in the adult and developing mammalian brain (Santagati et al. 1994, Azcoitia et al. 1999, Jordan 1999, Donahue et al. 2000, Kruijver et al. 2002, Platania et al. 2003). It is possible that low amounts of estrogen receptor in radial glial cells has escaped detection due to sensitivity of assay techniques (Pellegrini et al. 2005) and further studies are needed to examine the co-localization of aromatase and estrogen receptor subtypes in teleost fishes.

Estrogenic Modulation of Other Neural Signaling Systems

What neurochemical cues might lie downstream of estrogen in the pathway that controls sexual behavior? Effects of arginine vasotocin (AVT) and its mammalian homologue arginine vasopressin (AVP) on reproductive behavior are well documented in a broad range of vertebrate species including fish, amphibians, birds, and mammals (reviewed in (Goodson and Bass, 2001). As noted in the chapter describing neural aromatase immunoreactivity and co-regionalization with both AVT- and tyrosine hydroxylase (TH)-ir-, AVT is a neuropeptide of particular interest in the bluehead system. In studies using the bluehead wrasse in our lab, a clear positive association was established between AVT and social dominance independent of gonadal influences and TP males showed an increase in male-typical sexual behavior following administration of AVT (Semsar and Godwin, 2003, Semsar and Godwin, 2004, Semsar, Kandel and Godwin, 2001).

How might neurally produced estrogen affect AVT expression? In mice, ERβ has been co-localized with AVP in the hypothalamic paraventricular nucleus (PVN), the homologue to the magnocellular preoptic area (POA) of the hypothalamus in teleosts (Hrabovsky et al., 1998, Nomura et al., 2002). Studies in mice also suggest that ERβ expressed in the PVN may play a role in AVP regulation (Nomura et al., 2002, Shughrue et al., 2002, Somponpun and Sladek, 2002). Evidence for this effect includes the findings that estrogen treatment in wild-type mice significantly decreases AVP transcripts in the PVN, while these effects are not seen in ERβ knockout mice (βERKO) (Nomura et al., 2002). Human studies suggest an inhibitory role of estrogens in regulation of AVP expression in the supraoptic nucleus (SON; (Ishunina et al., 2000)), a key production site of AVP.

Interestingly, young women have significantly smaller AVP neurons in the SON than young men, although this difference decrease with age. Smaller AVP neuron size in young women is correlated with increased numbers of ERβ-ir neurons in the SON (Ishunina et al., 2000). Although we have not characterized their neurochemical phenotype, very large neurons in the portion of the teleost POA where AVT neurons are found do express an ERβ subtype (ERβb, Hawkins et al., 2005). An important next step in this work will be to determine whether these are AVT neurons as would be predicted from work in mice.

As discussed in Chapter II, monoamine neurotransmitters represent other neurochemical systems that can influence and be influenced by steroid hormone signaling. Behavioral studies in mice, quail, teleost fishes, and primates have demonstrated rapid steroid actions involving neurotransmitters (Cornil et al., 2006, Hull, Muschamp and Sato, 2004). The dopamine system regulates sexual behavior and function in a variety of vertebrates and is often responsive to the steroid hormone environment (see Balthazart, Baillien and Ball, 2002, Hull and Dominguez, 2005). Estradiol can rapidly increase the activity of tyrosine hydroxylase, the rate-limiting enzyme in catecholamine synthesis; the release and turnover of dopamine; and the number of DA re-uptake sites (see review in Cornil et al., 2006). Likewise, estradiol influences the serotonergic system by down-regulating the expression of 5-HT1A receptors in female rats (Osterlund and Hurd, 1998) and by increasing serotonin transporter mRNA and binding capacity in male rats (McQueen et al., 1999). In quail, estradiol treatment rapidly increased levels of the serotonin metabolite 5-hydroxyindoleacetic acid (5-HIAA) and 5-HIAA/serotonin ratios in the telencephalon and hindbrain (Cornil et al., 2006).

In another protogynous sex-changing fish, the saddleback wrasse, drugs affecting monoamines were used in an attempt to either induce gonadal sex change under non-permissive social conditions or prevent gonadal sex change under permissive social conditions. Increasing norepinephrine or blocking dopamine or serotonin led to sex change in experimental animals under non-permissive conditions. Increasing serotonin blocked sex change under permissive conditions. The results suggest that, in this fish, both dopamine and serotonin may be involved in preventing the initiation of gonadal sex change (Larson et al. 2003a). The behavioral effects of monoamines have only recently begun to be understood. In fish, reptiles, birds, mammals, primates, and humans, studies have suggested that serotonin is the primary regulator of aggressive behavior (reviewed in Summers et al. 2005). In sex-changing fishes, further studies in the saddleback wrasse linked a decrease in serotonergic activity in the raphe nucleus with behavioral sex change (Larson et al. 2003b). We explored the effects of manipulations of the serotonergic signaling system in our model, the bluehead wrasse. We used fluoxetine, a selective serotonin reuptake inhibitor, to effectively increase serotonin levels, and found that this decreased aggression in both lab and field experiments. (Perreault et al, 2003). This decrease in aggression was correlated with a roughly 50% decrease in AVT expression in the preoptic area of the hypothalamus (Semsar et al. 2004). The serotonergic system appears to be an integral part of the pathway involved in the conversion of a social cue into a neuroendocrine event, and it seems likely that the role of estrogens in mediating behavior may be influenced by monoamine neurotransmitters in a variety of neural systems that have yet to be explored.

Coexisting circuits for female- and male-typical behavior in the female vertebrate brain?

A recent study in mice demonstrated that 'knocking out' neural inputs of the vomeronasal organ (VNO) induces robust male-like sexual and courtship behaviors in female mice (Kimchi et al. 2007). The authors suggest that dual circuits controlling male and female-typical behaviors coexist in the female mouse brain and that their activity is controlled by environmental modulators (Kimchi, Jennings and Dulac, 2007). Similarly, in the bluehead wrasse and other sex-changing fishes, it appears likely that a functional male behavioral circuit is present prior to sex change. This is based on the observation that full behavioral transitions can occur in minutes to hours (Nakamura et al. 1989, Warner and Swearer 1991, Cardwell and Liley 1991, St. Mary 1993, Godwin 1994, Black et al. 2005). In seabasses such as the tobaccofish (*Serranus tabacarius*), the rapid alternation of sexual role is extreme as these fish are simultaneously hermaphroditic, meaning they possess both male and female sexual capabilities both internally and externally (Petersen 1995). The rapid nature of these changes suggests that circuits subserving both male and female sexual behavior are already present and their activity could be controlled by both endogenous and exogenous inputs.

Given the conserved nature of the neuronal circuits and networks that regulate social behavior in a variety of species (Newman, 1999, Goodson and Bass, 2002, Goodson, 2005), comparative studies in animals should also provide insights for clinical researchers. Social interactions are important to human health. Therefore, insights into how social influences affect neural function are becoming increasingly important in order to understand the role

social behavior plays in human mental illnesses such as affective and autism spectrum disorders (Caspi et al., 2003, Lesch, 2007, Lim, Bielsky and Young, 2005, Lord et al., 2000). Estradiol is considered to be a neuroactive steroid, a steroid that interacts with neurotransmitter receptors (Paul and Purdy, 1992). Neuroactive steroids could also have a role in the response to stress and the treatment of psychiatric disorders, such as depression, and, because they affect a broad spectrum of behavioral functions through their unique molecular properties, could influence novel therapeutic strategies in the treatment of psychiatric disorders (Rupprecht and Holsboer, 1999). Rupprecht & Holsboer (1999) list putative neuropsychopharmacological properties ascribed to neuroactive steroids including, among others, antidepressant, antipsychotic, and anxiolytic characteristics. The role of estrogen in the psychopathology of behavioral disorders is multifaceted, but the interrelationships between estrogenic regulation of the reproductive axis and key neural systems involved in behavioral affect offer exciting opportunities to explore the structure and function of behavioral systems in the brain that are potentially stress responsive (Cameron, 2004).

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