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SOCIAL INTERACTIONS OF *LYTHRYPNUS DALLI* AND THEIR EFFECTS ON  
AGGRESSION, NEUROPEPTIDES, STEROID METABOLISM, AND SEX-TYPICAL  
MORPHOLOGY

by

MICHAEL P. BLACK

Under the Direction of Matthew S. Grober

ABSTRACT

Social interactions can have profound effects on the behavior, physiology, and overall fitness of an individual. An example of this in *Lythrypnus dalli* is the removal of a male from a social group resulting in a dominant female fish changing sex. The dominant female's transformation involves a suite of changes including brain, behavior, morphology, and physiology. Following the social trigger (male removal), sex-changing individuals' morphology, steroid levels, and changes in the behavior were quantified in the field and compared to results found previously in the laboratory. There were lower rates of aggressive and courtship behavior in the field, but the change in behavior over time had a similar pattern and there were parallels in morphology and steroid levels between lab and field sex changers. The brains of dominant females also responded to social change. Aromatase, an enzyme that converts testosterone into estrogen, and oxytocin, a neuropeptide found in mammals, have been associated with vertebrate social and reproductive behavior. The fish homologue of oxytocin, isotocin, and aromatase are both found in *L. dalli*. Upon removal of a male from the social group, *L. dalli* dominant females experienced a decrease in the number of preoptic area isotocin-immunoreactive cells over the course of sex change (7-10 days) and a decrease in brain aromatase activity (bAA) levels within

hours, but not minutes, of male removal, while gonadal aromatase activity (gAA) decreased at a much slower time scale (beyond a week). Hours, but not minutes, after male removal, the sex-changing individual's bAA correlated with aggressive behavior increases and not the amount of time following male removal. Males that had just changed from female had different gonadal allocation and higher bAA levels than established males. Subordinate females had high gAA, but their bAA was between that of males and sex changers. In conclusion, dramatic changes in anatomy and neuroendocrine function can occur in response to social cues, individuals with similar reproductive behavior and external morphology can have large neuroendocrine and internal morphologic variation, and social interactions can affect steroid metabolism locally on a short time scale independent of gonadal modulation of steroids.

INDEX WORDS: estrogen, androgen, teleost, 11-ketotestosterone, reproductive, plasticity, CYP19, accessory gonadal structure, genital papilla, genitalia, challenge hypothesis

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A Dissertation Submitted in Partial Fulfillment of Requirements for the Degree of  
Doctor of Philosophy  
Georgia State University

2005

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## TABLE OF CONTENTS

	Page
ACKNOWLEDGEMENTS.....	iv
TABLE OF CONTENTS.....	vi
LIST OF FIGURES.....	viii
LIST OF ABBREVIATIONS.....	x
CHAPTER	
1 GENERAL INTRODUCTION.....	1
2 STEROID HORMONES AND BEHAVIOR ASSOCIATED WITH SEX	
CHANGE IN THE FIELD.....	12
Summary.....	12
Introduction.....	13
Methods.....	14
Results.....	24
Discussion.....	38
3 SOCIALLY-REGULATED SEXUAL DIFFERENCES IN PREOPTIC	
ISOTOCIN-LIKE IMMUNOREACTIVE CELLS.....	49
Summary.....	49
Introduction.....	50
Methods.....	52
Results.....	55
Discussion.....	59
4 SOCIALLY-REGULATED RAPID CHANGES IN AGGRESSION AND BRAIN	
AROMATASE ACTIVITY ARE CORRELATED.....	63
Summary.....	63
Introduction.....	64

Methods.....	65
Results.....	69
Discussion.....	76
5 RAPID BEHAVIORAL CHANGES PRECEDE CHANGES IN BRAIN	
AROMATASE ACTIVITY DURING SOCIALLY-MEDIATED SEX	
CHANGE.....	85
Summary.....	85
Introduction.....	86
Methods.....	87
Results.....	89
Discussion.....	89
6 GENERAL DISCUSSION.....	97
Variation in males.....	98
Social effects on brain aromatase activity and neuroendocrine modulation.....	101
Aromatase activity in fish and other vertebrates.....	104
Local brain changes independent of the gonads.....	106
REFERENCES.....	109
APPENDIX: ACKNOWLEDGEMENTS FOR PUBLISHED PAPERS.....	142

## LIST OF FIGURES

## FIGURE

1	Number of different species in each of the major vertebrate groups.....	3
2	Size-advantage model of sex change.....	6
3	Temporal sex change model for <i>Lythrypnus dalli</i> .....	8
4	Transverse section of bluebanded goby ( <i>L. dalli</i> ) gonad from a focal fish.....	23
5	Four representative behavioral profiles of <i>L. dalli</i> focal fish after the male has been removed.....	25
6	Temperature-corrected displacement and jerk behavioral profiles over the course of sex change for all <i>L. dalli</i> sex changers on artificial habitats.....	27
7	Scatterplot comparing mean ovarian and testicular allocations in transverse sections of <i>L. dalli</i> gonad in focal fish, males, and female.....	30
8	Scatterplot comparing percent testicular allocation in <i>L. dalli</i> gonad (testicular tissue size/ gonad size x100) to mean transverse cross-section size of testicular tissue and ovarian tissue in males, focal fish, and females.....	32
9	Scatterplot comparing percent testicular tissue allocation in <i>L. dalli</i> gonad (testicular tissue size/ gonad size x100) to genital papilla length to width ratio in focal fish, males, and females.....	33
10	Scatterplot comparing percent testicular tissue allocation in <i>L. dalli</i> gonad (testicular tissue size/ gonad size x100) to mean transverse cross-sectional area of gonad or AGS of focal fish and males.....	34
11	Scatterplot comparing <i>L. dalli</i> AGS interior wall thickness and genital papilla length to width ratio to the mean transverse cross-sectional area of AGS in focal fish and males.....	37

12	Free 11-ketotestosterone samples taken in the morning correlate with percent testicular tissue in the gonad, average accessory gonadal structure size, and last day displacements, but not genital papilla ratio in <i>L.dalli</i> .....	39
13	Jerk and displacement behavior of dominant males compared to the focal female prior to and in the first five days following male removal from the group in <i>L. dalli</i> .....	56
14	Sex change stage versus preoptic isotocin-immunoreactive cell number in <i>L. dalli</i> ...	57
15	Differences in preoptic isotocin-immunoreactive cell number and size between <i>L. dalli</i> females and males.....	58
16	The gonadosomatic index (gonad mass/body mass x 100) for <i>L.dalli</i> control females, dominance phase females, sex changers, males, and subordinate fish.....	70
17	<i>L.dalli</i> genital papilla (genitalia) length:width ratio at the beginning of the experiment and at sacrifice.....	71
18	Aggressive displacement behavior and aromatase activity in the brain and gonad of <i>L. dalli</i> .....	73
19	Correlation between aromatase activity and socially-induced increases in aggression in <i>L. dalli</i> during the dominance phase of sex change.....	75
20	Model for the potential neurosteroidal consequences of decreased aromatase activity in <i>L.dalli</i> .....	79
21	Pathways of glucocorticoid and androgen synthesis and inactivation in fish.....	83
22	The gonadosomatic index (GSI, gonad mass/body mass x 100) for control females, females that doubled their baseline displacements 10, 20, or 30 minutes after male removal, those females that went 30-min without doubling their displacements, control males, and sex changers.....	91
23	Displacement behavior on Day 4 (prior to male removal) and on Day 5, when the male	

was removed from all but the control group.....	92
24 Brain aromatase activity for control females and females sacrificed at 10, 20 and 30 min after male removal.....	93
25 Regressions of brain aromatase activity and baseline displacements, final displacements, and the increase in displacements (final-baseline displacements).....	94

## LIST OF ABBREVIATIONS

11-ketotestosterone (11-KT)

analysis of variance (ANOVA)

accessory gonadal structure (AGS)

arginine vasotocin (AVT)

aromatase activity (AA)

androgen to estrogen ratio (A:E ratio)

brain aromatase activity (bAA or BAA)

centimeter (cm)

CYP19 (cytochrome pigment 450 oxidase, family 19)

degrees Celsius (°C)

degrees of Freedom (df)

dehydroepiandrosterone (DHEA)

depth (D)

disintegrations per minute (dpm)

*exempli gratia*; for example (*e.g.*)

environmental potential for polygamy (EPP)

*Epinephelus merra* (*E. merra*)

estradiol (E2)

Figure (Fig.)

gonadal aromatase activity (gAA)

gonadosomatic index (GSI)

hour (hr)

hypothalamus-pituitary-adrenal, or in fish: hypothalamus-pituitary-interrenal axis (HPA axis)

hypothalamus-pituitary-gonadal axis (HPG axis)

*id est*, that is (*i.e.*)

isotocin (IST)

isotocin-like-immunoreactivity (IST-ir)

immunoreactive (-ir)

length (L)

length to width (L/W)

*Lythrypnus dalli* (*L. dalli*)

tricaine methanesulfonate (MS-222)

messenger ribonucleic acids (mRNA)

micromolar ( $\mu\text{M}$ )

micrometer ( $\mu\text{m}$ )

milliliter (ml)

millimeter (mm)

minute (min)

molar (M)

picograms (pg)

picomoles per hour per milligram (pmol/hr/mg)

poly vinyl chloride (PVC)

*Paralabrax nebulifer* (*P. nebulifer*)

*Porichthys notatus* (*P. notatus*)

*Rhinogobiops nicholsii* (*R. nicholsii*)

sample size for experimental or control group (n)

SRY-type high-mobility-group box (Sox)

standard error of the mean (SEM)

standard length (SL)

*Symphodus ocellatus* (*S. ocellatus*)

*Thalassoma bifasciatum* (*T. bifasciatum*)

*Thalassoma duperrey* (*T. duperrey*)

*Trimma okinawae* (*T. okinawae*)

versus (vs.)

width (W)



## CHAPTER 1: GENERAL INTRODUCTION

Social interactions can have profound effects on the behavior, physiology, and fitness of an individual. Reproduction can be highly socially regulated across taxa from social insects, like bees and ants, to vertebrates, such as naked mole rats (Ellis, 1995; Bloch *et al.*, 2002).

Examination of a full range of sociality suggests that highly social animals may respond more quickly and/or more dramatically to social disturbances (Summers, 2001). This idea makes sense in the short term, as an organism must respond to social cues or risk the repercussions of giving an inappropriate response (*e.g.* acting aggressive toward a much larger and stronger dominant animal and not changing that behavior quickly). By responding first to a social cue, an organism may benefit because its response can affect the actions of others in the group (Summers, 2001).

The idea also makes sense from an evolutionary perspective because if there is large reproductive benefit for properly assessing relevant social cues, selection should favor individuals with a neurobiology and behavior that better detects and responds to those social cues.

Primates, including humans, tend to be social species (Ghazanfar and Santos, 2004). As stressed by Insel and Fernald (2004), comparative studies with insight into the “social brain” may help the human condition through translational research to aid in researchers’ understanding of abnormal social cognition and corresponding deficits in social behavior as seen in neurodevelopmental disorders such as autism and schizophrenia and how social interactions reduce risk factors for medical disorders that are increased by social isolation and social separation (House *et al.*, 1988; Lord *et al.*, 2000). Understanding how social interactions cause

changes in the brain, what those changes are, and where they are located can help researchers to pinpoint deficits in social circuits and begin to target those deficits with novel therapies.

A comparative approach using a wider range of species than a few model organisms allows researchers to discover widely shared characteristics of brain organization that encompass these social circuits and to more reliably determine general principles of structure-function relationships in the nervous system that hold across species (Preuss, 2000). There are approximately 4,400 different species of mammals, and approximately 9,700 species of birds. Although this diversity may seem high, it is small compared to the diversity of fishes, with over 24,500 species, or over five times that of mammals (Fig. 1; Nelson, 1994; Pough *et al.*, 1996). Euteleost fishes account for most of the fish species, approximately 22,262 species, and include groups classically thought of as “fishes” including bass, halibut, perch, and salmon (Nelson, 1994). The high species diversity and rapid evolution of the euteleosts resulted in a high degree of phenotypic variation, a feature evident in the realms of phenotypic sex and social systems (Salzburger and Meyer, 2004; Volff, 2005).

Euteleost fishes have varied social systems and a wide range in sexual phenotypes from species with permanent sexes, such as cichlids, to species like bluehead wrasse that may change sex once in their lives. Other fish, such as the Japanese goby, *Trimma okinawae*, change sex multiple times within a lifetime. A range of phenotypic variation can also exist within a species, with numerous examples of species with multiple male morphs and the potential for multiple female morphs (Taborsky, 1994; Henson and Warner, 1997; Taborsky, 2001). For example, the ocellated wrasse, *Symphodus ocellatus*, exhibits four different male strategies in the same population (Taborsky *et al.*, 1987). Large males maintain nests and care for eggs, while small males are parasitic spawners (sneakers). Mid-sized males, called satellites, help the large

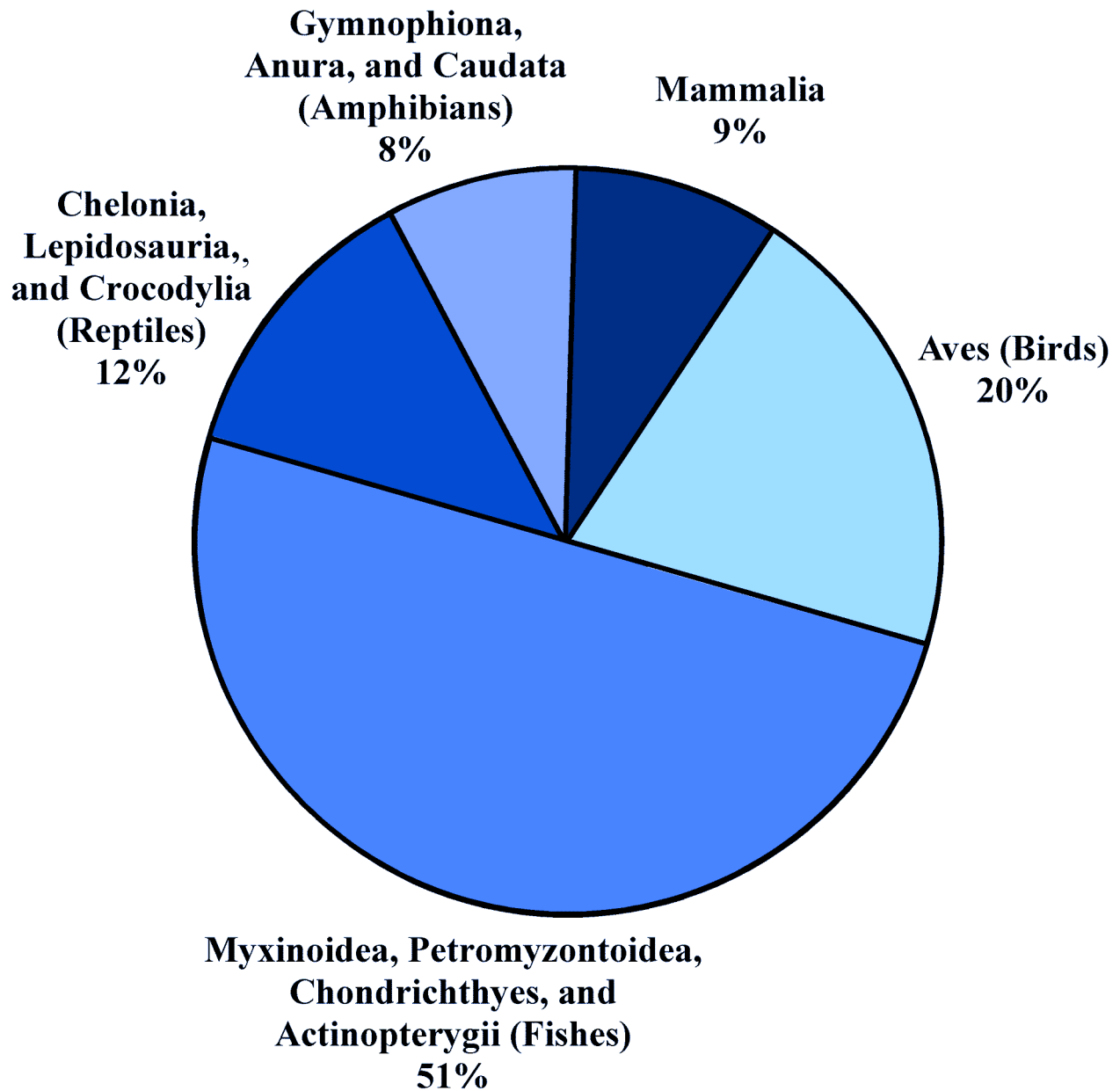


Figure 1: Number of different species in each of the major vertebrate groups as approximate percentages of the total known vertebrates (Reprinted with permission from Black and Grober, 2003).

males by defending nests against sneakers in exchange for the occasional chance to court and spawn with females. The largest males, dubbed ‘pirates,’ dominate over nesting males and take over their nest sites. In some cases, particularly in other *Symphodus* species, pirates fertilize eggs in the nesting males’ nests and leave them ‘under the care’ of the nesting males (van den Berghe, 1988; Taborsky, 1994). Females also can show variation in sexual phenotype. In the bluehead wrasse, *Thalasomma bifasciatum*, females express two mating tactics: group spawning and pair spawning. Both of these tactics occur within a single population. As with male strategies, female strategies seem to be size dependent: the largest females only spawn with one male at a time, while smaller females primarily spawn in groups (Warner and Hoffman, 1980; Warner, 1984a; Warner, 1985). The variation in sexual phenotypes for males and females of *S. ocellatus* and *T. bifasciatum* exemplifies the diverse alternative reproductive tactics used by fish, and there are many more examples among other fish species (for comprehensive reviews of reproduction, sexuality, and social systems in fishes, see Breder and Rosen, 1966; Taborsky, 1994).

To understand evolutionary pressures that may be driving nervous system structure-function relationships, phenotypic plasticity, and the context of the social system, it is also helpful to have an idea of the evolution behind a particular social system. In this regard, the environmental potential for polygamy (EPP) provides a useful organizing principle for understanding the evolution of diverse sexual phenotypes and mating systems that affect and are affected by social groups. The EPP describes the degree to which resources are distributed or aggregated, such that a limited number of individuals can control access to a large amount of required resources and, as a result, monopolize mates who seek those resources (Emlen and Oring, 1977). For instance, male monopolization of mating resources results in polygyny, or the acquisition of multiple females by a limited number of top-ranked males. Because of this, there is tremendous pressure

to acquire resources and a resultant skew in male reproductive success, which greatly benefits the monopolizers. Those largest in size usually are better able to monopolize necessary resources and mate with multiple females because of the monopolization. Thus, particularly in many fishes, there is evolutionary pressure to become male when large. This evolutionary argument for why a fish would change sex is explained by Ghieslin (1969) and further illustrated by Warner (1975, 1984b) (see Fig. 2). A fish species that can increase its reproductive success by changing from one sex to the other during its lifetime will be under evolutionary pressure to do so and the ability of its gonads to develop into testicular or ovarian tissue, or the gonad's bipotential nature, allows the fish to do this. Sex change has evolved multiple times in the evolutionary history of fishes, and has been reported in over 27 different families of fish (Devlin and Nagahama, 2002).

Socially regulated sex change is of particular interest in understanding how social interactions can affect the brain because of the dramatic changes in brain, behavior, morphology, and physiology that occur. This research focuses on the bluebanded goby, *Lythrypnus dalli*, to explore this dramatic observable transformation that results from social interactions.

Dominant and subordinate relationships or social dominance hierarchies are important components of socially regulated sex change. There is a range of possible reproductive outcomes when an animal is dominant over another (reviewed in primates by Sapolsky, 2004). Examples in subordinate fish can include delayed maturation, sneak spawning strategies, or inhibition of sex change or morph change within the same sex into a new morph (Davis and Fernald, 1990; Taborsky, 1994; Fox *et al.*, 1997; Taborsky, 2001). Like many socially-regulated sex-changing fish (reviewed in Shapiro, 1979, and Ross, 1990), *L. dalli* shows inhibition of sex change in the presence of a dominant male (Reavis and Grober, 1999). Unless there is a subordinate female conspecific, sex change will not occur (Carlisle *et al.*, 2000; Rodgers *et al.*, unpublished).

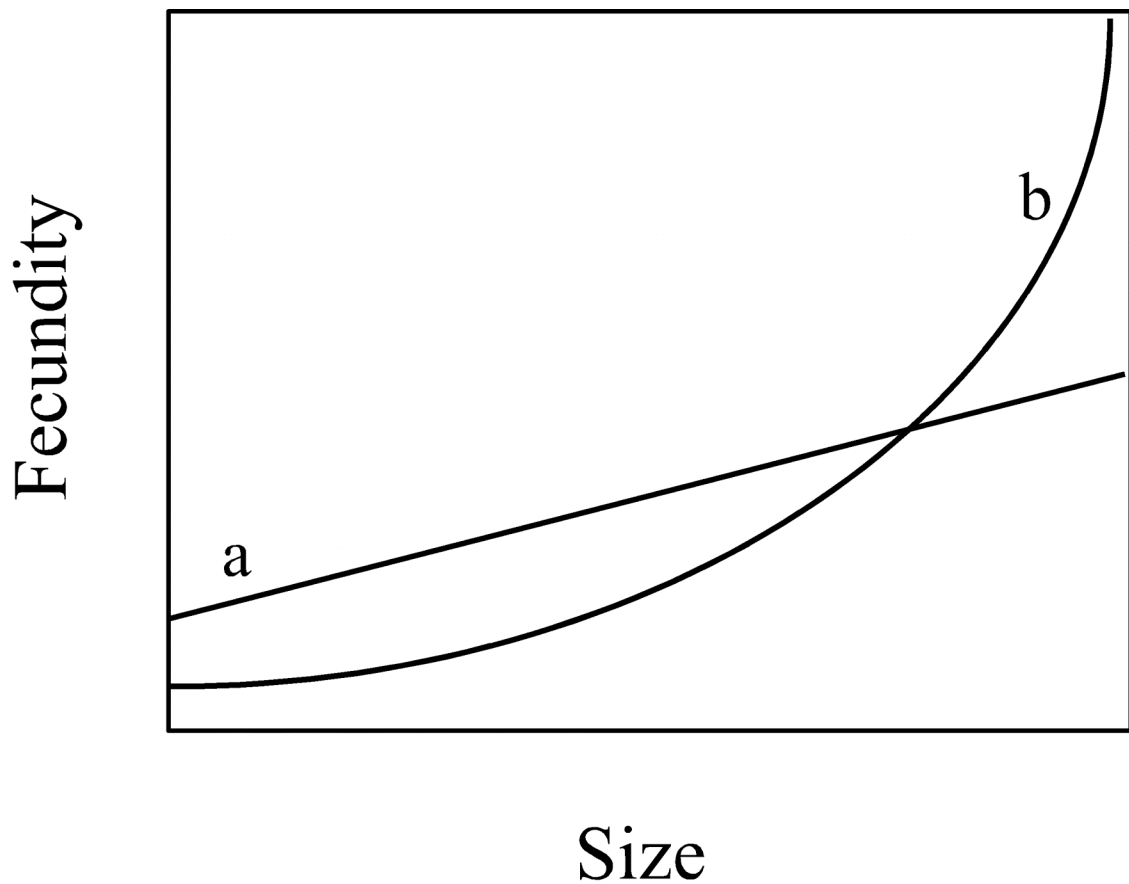


Figure 2: The size-advantage model of sex change (printed with permission from Warner, 1975) shows (a) a fish whose fecundity increases linearly with size (*e.g.* females that are able to produce more eggs with larger body size) and (b) a fish whose fecundity exponentially increases at a very large size (*e.g.* for a male that only at a very large size can successfully compete for and defend a nest resource, permitting mating with multiple females). Lines a and b intersect where a fish would have great advantage in changing sex (in the example, from female to male).

Most of the small fish in a population of *L. dalli* are female, although at the beginning of a reproductive season, anatomical evidence suggests that a low percentage (<10%) of the population may be small, sneak spawning males (Drilling and Grober, 2005).

*L. dalli* is a good species to study in the field because the fish can be very common, living in densities of up to 120/m<sup>2</sup> across its range, from the Gulf of California, Mexico, to Morro Bay, California, at depths ranging from intertidal to 64 meters deep (Miller and Lea, 1976; Wiley, 1976; Behrents, 1983; Steele, 1996). *L. dalli* readily shows social behavior and reproduces in the lab in small aquaria due in part to its small size and benthic nature (Reavis and Grober, 1999). *L. dalli* also tends to be site-specific in the field, with social groups aggregating around long-spined sea urchins, *Centrostephanus coronatus*, where they can evade predators by going between the urchin spines (Behrents, 1983; Hartney and Grorud, 2002), or on rocky vertical faces that have crevices in which to hide (Wiley, 1976). Sex ratios observed in populations ranged from 0.43:1 to 4:1 females to males with the highest frequencies of females during the reproductive months of the summer (Wiley, 1976; Behrents, 1983; Drilling and Grober, 2005).

Reavis and Grober (1999) conducted laboratory experiments with groups of fish that had four female fish newly exposed to each other without a male or males removed from a group of one male and three to five females. The sex-changing individual from each group was observed for two behaviors, displacements and jerk swims. Briefly, displacements are when a fish moves within 5 cm of another fish, causing the other fish to move away. Jerks are a male-typical courtship behavior characterized by a distinctive approach toward another fish with abrupt starts and stops and erect fins. Out of the collective behavioral observations of the dominant fish changing sex, a behavioral model for sex change was developed (Reavis and Grober, 1999; Fig. 3).

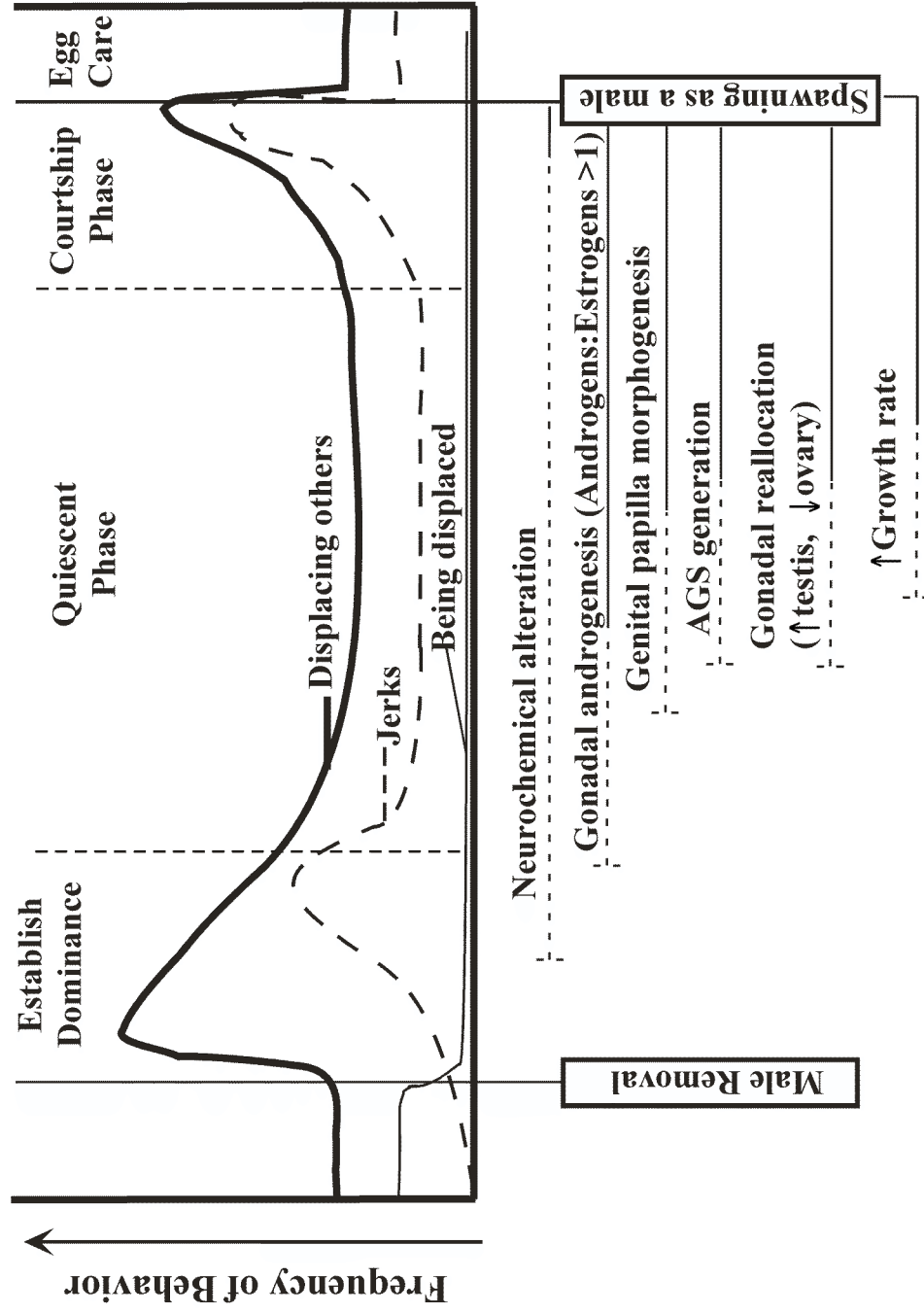


Figure 3: Temporal sex change model for *Lythrypnus dalli* showing frequency of displacing and jerking behavior of a focal female from before male removal until spawning as a male (modified from Reavis and Grober, 1999 with kind permission of Springer)



During sex change, this behavioral model has a profile that is useful in pinpointing time points at which changes in the brain, anatomy, and physiology of *L. dalli* occur relative to behavioral changes (see later chapters of this dissertation). Although the groups were similar in composition to those found in the field (Drilling and Grober, 2005), it was unknown if *L. dalli* would show a similar behavioral profile in the field. It is important in studying the neural mechanisms of social circuits to study social interactions that are naturally relevant to the study organism, so that the information obtained gives insight into the biology of the organism and is not just simply an inappropriate artifact of the behavioral assay (Ghazanfar and Santos, 2004; Insel and Fernald, 2004). The first chapter of this dissertation tests Reavis and Grober's (1999) behavioral model, observing how male removal affects dominant females in the field and also quantifies changes in morphology and physiology for comparison to changes found in the lab.

Some of the neural and hormonal mechanisms associated with behavioral changes in response to social circumstances appear to be conserved among the vertebrates, and those factors that differ can provide insight into alternative mechanisms in appropriate response (Insel and Fernald, 2004; Woolley *et al.*, 2004). Some of the same hormones and peptides active in mammals have similar effects in birds, reptiles, amphibians, and fish (Goodson and Bass, 2001; Woolley *et al.*, 2004). For instance, the vasopressin/oxytocin system has been associated with social and reproductive behavior across vertebrate taxa (Gimpl and Fahrenholz, 2001; Goodson and Bass, 2001). Likewise, a region of the brain known as the preoptic area has been implicated in social and reproductive behavior in vertebrates (Nelson, 2000; Balthazart *et al.*, 2004). Virtually all vertebrate species have an oxytocin-like and vasopressin-like peptide (Gimpl and Fahrenholz, 2001). Further, there tends to be a sex difference in vasopressin-like peptides with higher levels, particularly in the preoptic area, in males (Goodson and Bass, 2001). The

vasopressin homologue in fish (Murphy *et al.*, 1998), vasotocin, has this pattern in *L. dalli*, as forebrain vasotocin-immunoreactive cell size significantly increases as a fish changes from female to male (Reavis and Grober, 1999). A neuropeptide in the same family, oxytocin, in mammals generally facilitates reproductive behavior and is also associated with social behavior, particularly in females (Gimpl and Fahrenholz, 2001). Isotocin is the fish homologue to oxytocin in mammals (Murphy *et al.*, 1998). Although there is much information on vasotocin in fishes, little is known about isotocin in the brain of fishes and how it relates to behavior. The second chapter investigates the affect of male removal from a social group on the dominant female's isotocin-like (IST) neuropeptide in the preoptic area of the brain.

Steroids can affect neuropeptide anatomy in the brain, and steroid modulation, particularly by aromatase, has been implicated in sexual differentiation (Balthazart and Ball, 1998; Goodson and Bass, 2001). Chapter 4 covers the effects of male removal on changes in the activity of aromatase, the enzyme that converts testosterone into estradiol, in both the brain and the gonad of the dominant female *L. dalli*, both early in the sex change process and at its completion. Aromatase has been implicated in the feedback of steroid hormones on gonadotropin secretion, activation of male sexual behavior, and brain sexual differentiation (reviewed in Balthazart and Ball, 1998). As such, it makes sense that a social interaction causing sex change would have an effect on aromatase activity and that these changes might happen early on in the sex change process.

The fourth chapter is a follow-up study testing a hypothesis derived from the third chapter, investigating whether brain aromatase activity was decreasing fast enough to affect the increases in aggressive behavior following male removal. This study's behavioral sampling was on a much shorter time scale, looking for changes within minutes after male removal, and in this

experiment, fish early in the process of sex change are sacrificed within the first 30 minutes following male removal to look for rapid changes in both behavior and aromatase activity.

All of these chapters are tied together by an interest in understanding how a change in the social environment (removal of a male from a group) influences the brain, behavior, and morphology of *L. dalli*. The goal of this study is to use natural changes following removal of a male and examine the potential brain mechanisms involved to understand how social interactions can affect the behavior, brain, steroid synthesis, and morphology of an organism. Many brain chemicals and signaling pathways share similarities among vertebrates (*e.g.* Grober, 1997; Goodson and Bass, 2001). By understanding the changes in the brain and behavior of a sex-changing fish, researchers can better understand how social interactions affect sexual function at a mechanistic level and find interesting implications for other organisms by comparing similarities and differences across species.

## CHAPTER 2: STEROID HORMONES AND BEHAVIOR ASSOCIATED WITH SEX CHANGE IN THE FIELD

Figures and text modified with permission from: Black, M.P., Moore, B., Canario, A.V.M., Ford, D., Reavis, R. H., and Grober, M.S. 2005. Reproduction in context: Field testing a lab model of socially controlled sex change in *Lythrypnus dalli* (Gilbert). *Journal of Experimental Marine Biology and Ecology* 318:127-143.

Author roles: M.P. Black, B. Moore, D. Ford, R.H. Reavis, and M.S. Grober designed the experiment. M.P. Black and B. Moore wrote the manuscript. M.P. Black, B. Moore, and D. Ford collected preliminary and experimental data and analyzed the data. A.V.M. Canario ran the steroid assays.

### Summary

Social interactions can have profound effects on reproduction and the proximate mechanisms involved are just beginning to be understood. *Lythrypnus dalli*, the bluebanded goby, is an ideal organism for analyzing the dynamics of socially controlled sex change both in the lab and field. As with most research species, the majority of its behavioral and physiologic study has been performed in the laboratory. The goal of my study was to induce sex change of *L. dalli* in a more natural environment and compare field dynamics with Reavis and Grober's (1999) laboratory-based model. Groups of *L. dalli*, composed of one large male and three females of varying sizes were introduced into artificial habitats in the field. After male removal, the dominant, largest female underwent protogynous sex change in the majority of the groups.

Within fifteen days, nine out of fifteen of the dominant females (focal fish) successfully fertilized eggs as males, compared to 13 out of 17 in the lab. Focal fish displayed the distinctive temporal sequence of behavior changes consisting of a dominance, quiescent and courtship phase. In addition, focal fish had gonads, genital papillae, and accessory gonadal structures with morphology in between that of females and males. Those fish that fertilized eggs had this transitional morphology, but were functionally male. Steroids of focal fish were assayed by water sample, and morning samples of free 11-ketotestosterone (11-KT) positively correlated with the percent of male tissue in the gonad, with the size of the accessory gonadal structure but not the genitalia (genital papilla) and with aggressive displacement behavior on the last day before the fish were sacrificed. These morphological, physiological and behavioral patterns parallel those seen in the lab. Lower rates of behavior and the dramatic effects of ambient temperature in the field provide insights as to how the environmental context modifies the behavior and, subsequently, the reproductive function of individuals within a social group.

## **Introduction**

Sex change from female to male, protogyny, is the most common form of hermaphroditism in coral reef fish (Warner, 1984b). In many protogynous fish, sex change is mediated through social interactions with other fish. Removal of the dominant male and the presence of a subordinate female trigger a newly dominant female to change sex, restructuring the brain, behavior, gonads, hormones, genitalia, and secondary sex characteristics (*e.g.*, Robertson, 1972; Shapiro, 1981; Ross *et al.*, 1983; Ross, 1984; Nakamura *et al.*, 1989; Godwin *et al.*, 2000; see also Fig. 3). *Lythrypnus dalli*, the bluebanded goby, follows this pattern, with

removal of the dominant male from a social group resulting in sex change only if a female becomes the dominant and has a female subordinate to her (Reavis and Grober, 1999; Carlisle *et al.*, 2000).

Lab studies of *L. dalli* have investigated behavior during sex change and hormonal effects on genitalia, gonads and secondary sex characteristics, but all of these changes have not been observed in the same sex-changed fish and integrated with individual hormone levels under field conditions. As behavior and hormones are key regulators of sex change, this study will duplicate a detailed behavioral and morphological analysis of the sex change process to determine if conclusions made about *L. dalli* from lab experiments are valid in a more natural marine environment. In addition, both laboratory and field studies suggest some reproductive traits (*e.g.* accessory gonadal structure and behavior, Reavis and Grober, 1999; Drilling and Grober, 2005) may be more insightful than others (*e.g.* gonad and genitalia, St. Mary, 1993; St. Mary, 1994a; Carlisle *et al.*, 2000) in determining functional sex, and this study will compare those traits with regard to functional sex.

## Methods

### *Natural History*

*L. dalli* behaves primarily as a sequential protogynous hermaphrodite, however it is capable of sex change in both directions (St. Mary, 1993; Reavis and Grober, 1999). This small fish (standard length 18-45 mm) commonly inhabits rocky reefs from Morro Bay, California, to the Sea of Cortez (Miller and Lea, 1976). *L. dalli* are planktivores (Hartney, 1989) and live in

mixed sex groups averaging from 1.10:1 to 4:1 females to males during the reproductive season with densities up to 120/m<sup>2</sup> (Eckert, 1974; Wiley, 1976; Behrents, 1983; St. Mary, 1994b; Steele, 1996; Drilling and Grober, 2005). Spawning primarily from April to September, males externally fertilize eggs from multiple females in their group and provide all parental care to the eggs (St. Mary, 1993; St. Mary, 1994b).

### *Experimental Paradigm*

Seven artificial fish habitats were constructed, each composed of a 20.3 cm square cinder building block that had been partially filled with cement to create a cube with a single 15.2 cm square cavity on one of its faces. Each block was cemented to a 30.5 cm by 30.5 cm cement paving tile with the cavity facing sideways at a slight upward angle. A 15.2 cm long by 1.9 cm diameter PVC tube was attached to the top of the block and to the front of the tile base. To serve as a suitable but removable nesting site, glass test tubes were inserted into the PVC until their rims were flush with the PVC opening. *L. dalli* has been reported to lay and fertilize eggs in previous uses of this arrangement (St. Mary, 1994b; St. Mary, 1996). The habitats were placed on a reef in approximately 10-meters of seawater at Bird Rock, Santa Catalina Island, California in March of 1999 so that they would be encrusted with natural organisms. Two months later, the habitats were relocated approximately 350 meters from Bird Rock to a 10-meter deep, sandy bottom in Big Fisherman Cove (33°28' N, 118°29' W), near the Wrigley Institute for Environmental Studies (WIES). The habitats were placed five meters apart in two parallel lines, one line with three habitats and the other with four. Each cavity was oriented away from other habitats and facing open sand. An appropriately sized sea urchin, *Centrostephanus coronatus*,

was added into the cavity of each habitat, as *L. dalli* use urchins as a central defensive point and refuge from predators (Hartney and Grorud, 2002).

*L. dalli* were collected in the waters around Bird Rock using 10% quinaldine sulfate (Sigma Chemicals) in acetone and dip nets (California Fish and Game Permit No. 802013-01). After collection, fish were transferred to large, flow-through seawater tables located on the WIES waterfront. For processing, fish were anesthetized using MS-222, tricaine methanesulfonate (Sigma Chemicals). Standard length of the fish was measured ( $\pm 1$  mm) and the fish were visually sexed under a dissection microscope based on genital papilla length to width ratio. The genital papilla is primarily sexually dimorphic, but only loosely correlates with testicular tissue allocation in the gonad. Fish with female-typical genital papillae have greater than 95% ovarian tissue (St. Mary, 1993). However, fish with male papilla morphology may have between 5-100% testicular tissue, so it is an imperfect diagnostic of sex (St. Mary, 1993). During protogynous sex change, the papilla is rearranged in a process of elongating and narrowing (Reavis and Grober, 1999). Fish with questionable papillae were excluded. Individuals were uniquely identified by the number of bands on each side, distinctive gaps in bands and dorsal striping (see Reavis and Grober, 1999).

Groups were made throughout the summer starting on May 25, 1999 and consisted of a large male ( $>30$  mm), a large female ( $28.29 \pm 1.21$  mm), a medium female ( $24.47 \pm 1.01$  mm), and a small female ( $22.78 \pm 1.42$  mm). With the exception of the small female, each fish was  $>3$  mm smaller than the previous fish in the grouping. There were two exceptions to these criteria: in one group, there were four females instead of three and, in another group, the large dominant that was removed was female, not male. In both cases these fish changed sex, but neither was included in the behavioral analysis. Fish with different markings were grouped for easy visual



identification, sealed into ziplock bags of seawater for transport, and introduced to individual habitats.

For four days following introduction, the groups were allowed to acclimate and establish a social hierarchy. One day prior to male removal, groups were observed to confirm that the male was dominant in the group. Five days after introduction of the group, the male fish in each habitat was removed using dip nets and strategically placed squirts of quinaldine sulfate from a syringe. Any nesting tubes containing eggs were replaced with empty tubes.

The males were removed from all groups because sex change in artificial habitats had been shown in the past and behavioral observations of fish groups with a male remaining present had been made in the laboratory and field without the lab-derived behavior pattern or sex change occurring (St. Mary, 1994a; Black, Reavis and Grober, unpublished). Concurrent with this experiment, following male removal from natural field groups, daily 10-min behavioral observations were done at the Bird Rock location. Some of these natural field groups had a male immigrate in and prevent sex change of the female. These data are included for comparison. At the artificial habitats, no evidence of emigration to or immigration was observed. Fish introduced to the artificial habitats remained solely in and around the safety of the urchin.

### *Behavioral Observations*

Following male removal, two 15-min observations, starting at 0900 and 1500 hours, were made daily to monitor the behavior of individuals in each group. After the diver slowly approached the habitat, the fish were given two minutes to acclimate while the diver ascertained the identity of all of the fish in the group by size and distinctive banding/markings. Observations

were consistent with Reavis and Grober (1999) for comparison to the behavioral profile observed in the lab (Fig. 3). Briefly, behavior of the large female (focal fish) was categorized and recorded as approaches, being approached, displacements, being displaced, and jerks. An approach was defined as the focal fish moving to within 5 cm of another fish, approximately two body lengths. If the approach caused the approached fish to move away, then the behavior was termed a displacement. If another fish performed this behavior toward the focal fish it was recorded as approached by and displaced by, respectively. Jerks are a male-typical courtship behavior characterized by a distinctive approach toward another fish with abrupt starts and stops and erect fins. On day zero, the male was removed in the morning, and the focal fish's behavior was only recorded in the afternoon. All following days, the morning and afternoon behavior were averaged to calculate a daily rate of behavior until the day of focal fish removal. The same procedures for observations were followed in groups on natural field groups at the Bird Rock location, except groups were only observed once per day for 10 min and there was an additional assessment of feeding behavior, as determined by bites into the water column. Due to low light in the cavity of some habitats, the observer occasionally used a flashlight to illuminate the rear of the cavity in an area near the fish, casting indirect light on the observed fish.

After each observation, nesting tubes at the habitats were checked for eggs by slowly pulling the test tube from the PVC sheath, visually examining, and then reinserting it. If eggs or embryos were present, they were visually inspected for eyes to ascertain fertilization. The groups were considered complete if fifteen days following male removal had passed, or if fertilization had occurred. Fifteen groups went to completion. Four of these groups had no eggs in the nest tube fifteen days after male removal, eleven habitats had eggs in a nest tube, and nine of those had egg clutches that showed developed embryos, confirming fertilization. The following

analyses focuses on two sets of fish, the large females from those habitats that ran to completion, called focal fish ( $n=15$ ), and a sub-set of the focal fish, sex changers ( $n=7$ ), that originally had a male and two subordinate fish and successfully fertilized eggs as a male. The groups that had three subordinate fish or had a dominant female removed were not included in this sex changer classification because of potential confounds on the behavior and timing of sex change.

At the end of all observations and egg checks, water temperature was recorded using a Suunto Solution  $\alpha$  digital dive computer. These data were combined with the 10-meter depth daily temperatures from the nearby WIES pier to compile daily water temperature for the site over the course of the study. During the experiment, the water temperature ranged from 15.6°C in late May to 20.1°C in late July. Therefore, different groups of fish were exposed to increasing water temperatures as the summer progressed. Fertilized eggs were laid from 11 days after male removal in cooler temperatures to 4.5 days in warmer temperatures. The amount of time from male removal until fertilized eggs were laid negatively correlated with the average water temperature experienced by the particular habitats (ANOVA,  $r^2=0.658$ ,  $F_{1,5}=9.6$ ,  $p=0.027$ ) indicating that, as temperature increased, the latency to fertilized eggs and presumably the time to change sex decreased. Therefore, to create a composite behavioral profile of sex changers, correction for temperature variation was required.

Temperature variation between the groups was controlled for by aligning their endpoints (the day each sex changer fertilized eggs as a male). Behavior profiles were matched in length of time by elongating the shorter behavioral profiles of fish that changed sex in warm water to match the longest behavioral profiles of fish that experienced colder water (11 days). Because Reavis and Grober (1999) found that the quiescent phase was the most temperature sensitive phase, days were added to the middle to prolong shorter time groups. Half of the data points

were placed at the beginning and half of the data points were placed at the end. If there was an uneven number, the middle day was added on to the first half. For the spaces in the middle of the shorter behavioral profiles, no data were used, resulting in lower sample sizes for the middle periods. Because of this method, sample sizes were low for statistical tests, but without using this method, the variation was too high due to temperature differences.

Experiments continued from May 27<sup>th</sup> to July 29<sup>th</sup>, 1999. Observations of each group of fish were terminated either when embryos with eyes were present in the nesting tube or fifteen days after male removal. If the large female disappeared or was the only fish remaining, the group was collected, identified, and examined. During the following observation, a new group of four fish including a new male was introduced into the vacant habitat and the process was repeated.

#### *Hormonal and anatomical analyses*

When 15 days elapsed or embryos with eyes were found, the fish in the habitat were recaptured, re-measured and sexed by genital papilla. The large female was placed in a beaker containing 50 ml of seawater for one hour to collect excreted urine. Steroids found in urine have been shown to correlate with plasma steroid levels (Scott and Liley, 1994). The water was frozen until processed for radioimmunoassay and assayed for 11-ketotestosterone (11-KT) and estradiol (E2) (for details, see Carlisle *et al.*, 2000). Six fish were processed after the morning observation (AM) and nine after the afternoon observation (PM). Finally, large fish were euthanized using excess dissolved MS-222 and fixed in 4% paraformaldehyde after removal of the eyes to facilitate fixation of the brain.

The genital papilla of each preserved fish was measured by capturing a uniform 25X-magnification image of the ventral region with a dissecting microscope. The image was displayed on a monitor and the dimensions were measured using a ruler, from which the length to width (L/W) ratio was calculated. The torsos containing the gonad were sunk in a 30% sucrose solution, serially sectioned (30  $\mu$ m) on a cryostat from anterior to posterior and mounted on chrom-alum coated slides. Tissues were stored at  $-20^{\circ}$  C until hematoxylin-eosin staining (Presnell and Schreibman, 1997).

Using NIH Image 1.62a (W. Rasband, NIH, Bethesda, MD), the 200X magnified images of a series of transverse sections of gonad for each fish were analyzed for the areas of testicular and ovarian tissues. The testicular and ovarian areas were averaged for all sections of the entire gonad for an average percent allocation of testicular and ovarian tissue. Percent testicular tissue was determined by dividing testicular tissue by total gonadal tissue. The presence of spermatozoa (tailed sperm) in the testicular regions was noted (as in St. Mary, 1993).

If an accessory gonadal structure (AGS) was present, the same imaging technique was used to calculate its area. The AGS is a pair of multi-chambered lobes containing sperm as well as mucins and/or steroid derivatives that are characteristically male (Miller, 1984; Cole and Robertson, 1988; Fishelson, 1991; Cole *et al.*, 1994; Scaggiante *et al.*, 1999). The AGS structure is similar to the seminal vesicles and sperm duct glands found in other fish species (reviewed in Lahnsteiner *et al.*, 1992) but differs in that it originates from the ovarian wall rather than the sperm ducts (Cole and Robertson, 1988). Images of the AGS and the partitions between its chambers were captured to evaluate sperm content and measure partition thickness along the central region of a bisecting midline throughout each AGS (Fig. 4). An average columnar epithelial wall thickness was calculated for the AGS of each fish for an estimate of the wall

thickness between partitions. The amount of sperm seen within each AGS section was ranked as: 0 = no appreciable sperm visible, 1 = small sperm content forming sparse, infrequent sperm aggregations, 2 = large amounts of aggregated sperm. The average amount of sperm of all AGS sections was rounded to a whole number, characterizing the AGS sperm content.

For comparison to focal fish and sex changers, five male (standard length, SL,  $34.2 \pm 1.3$  mm) and five female fish (SL  $23.4 \pm 0.9$  mm) were taken and preserved from concurrently running experiments using fish in seawater tables on the WIES waterfront that had similar group composition to those in the field. Sex was predicted by size, observation of sex-appropriate behavior, and papilla L/W ratio. Poor fixation and sectioning of the male fish prohibited reliable gonad analysis of the male fish, so data were collected from preserved male fish (SL  $32.6 \pm 1.9$  mm) that had been in large group tanks. These fish had exhibited male behavior and had male morphology. After histology was performed, all of these representative male and female fish had gonads consistent with their predicted sexes.

### *Statistical Analysis*

Data were analyzed using SPSS 8.0 (Chicago, IL) and JMP 5.0 (Cary, NC). Simple linear regression was used to test relationships between data. Normally distributed data were analyzed using t-test and ANOVA analysis. For data that did not distribute normally, the Mann-Whitney and Kruskal-Wallis tests were used as nonparametric alternatives. A repeated measures ANOVA was used to compare behavioral data over time. For the jerk behavior, the temperature-corrected data were clumped by averaging values among each of the following time points: the first day following male removal (day 0), days 1-3 (dominance), days 5-8 (quiescence), and days 9-11

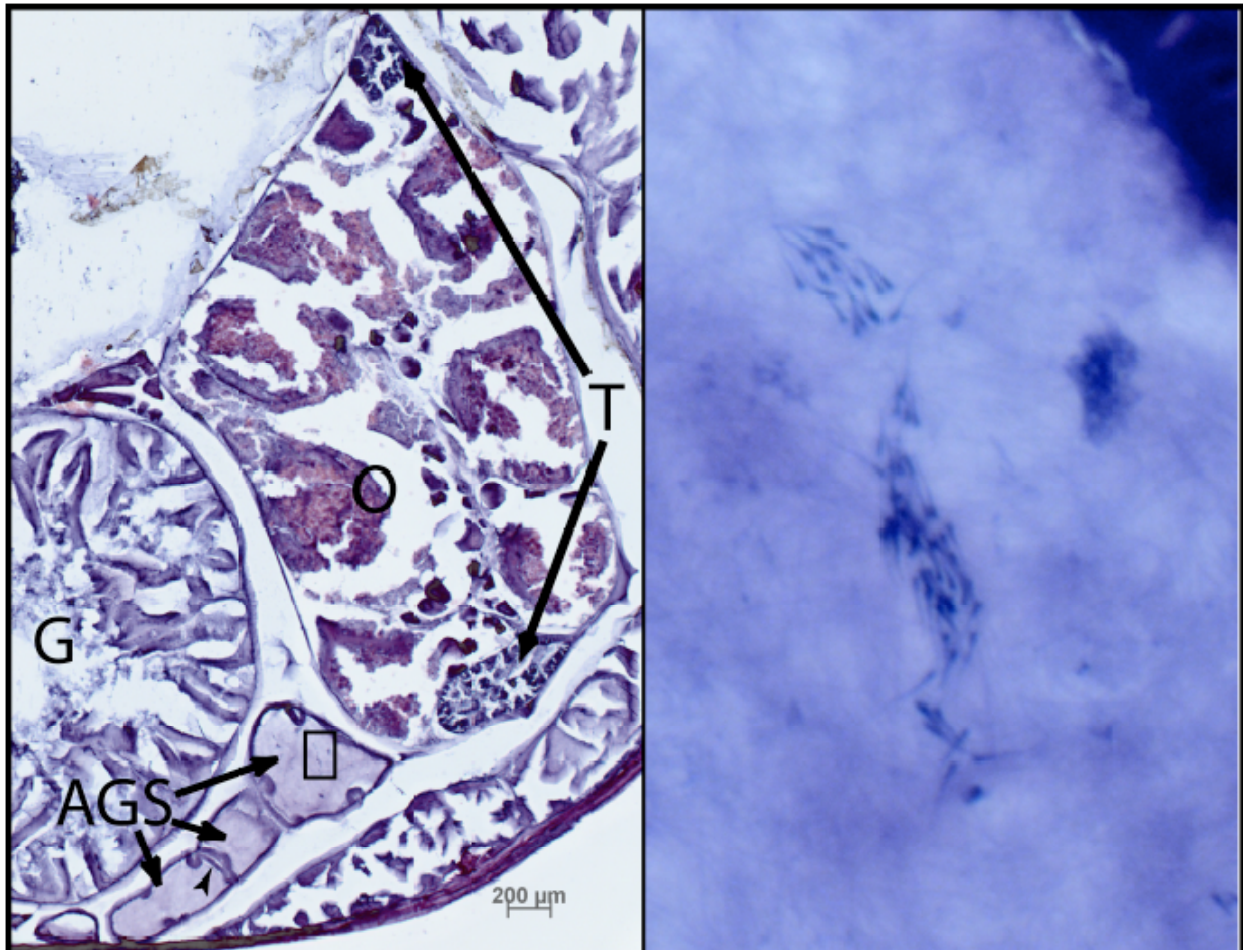


Figure 4: Transverse section of bluebanded goby (*Lythrypnus dalli*) gonad from a focal fish with 7.5% testicular tissue (T), 92.5% ovarian tissue (O), and a multi-chambered accessory gonadal structure (AGS) containing sparse crypts of sperm (rectangle within AGS enlarged to the right to show sperm). Arrowhead points to AGS wall. Gut (G) labeled for reference. Scale bar length = 200  $\mu\text{m}$ .

(courtship). The data were then transformed by the 2/3 power for normality, and compared using linear contrasts. Data are reported as means  $\pm$  SEM.

## Results

### *Behavior*

When the male was removed from each field habitat, the focal fish was rarely displaced by others, and approaches by the focal fish toward smaller females resulted in their displacement over 99% of the time. Analysis of jerk and displacement rates yielded common behavioral profiles for sex changers. As seen in sample behavioral profiles from two of the sex changing fish (Fig. 5A,B), after male removal the focal fish established dominance through frequent displacement of other fish. In sample profile one (Fig. 5A), displacements first peaked on day 2. Concomitantly, the fish began jerking behavior that peaked on day 2. On days 5 through 8, both displacements and jerking subsided, followed by an increase of both on day 9. The jerking was associated with displays leading the females toward the nesting site. On day 11, eggs were laid in the nesting tube and were subsequently fertilized. After initial spawning, this fish displayed egg care while continuing to court the other female in the group.

Sample profile two (Fig. 5B) shows a shorter time to change sex (only five days). As in profile one, profile two shows the same high, low, then high pattern of displacement frequency. Additionally, the rate of jerks and displacements increased in association with eggs being laid.

Comparing the sample behavior profiles, the sex changing fish in profile one experienced an average temperature of 16.3°C and took 11 days to complete functional sex change. In



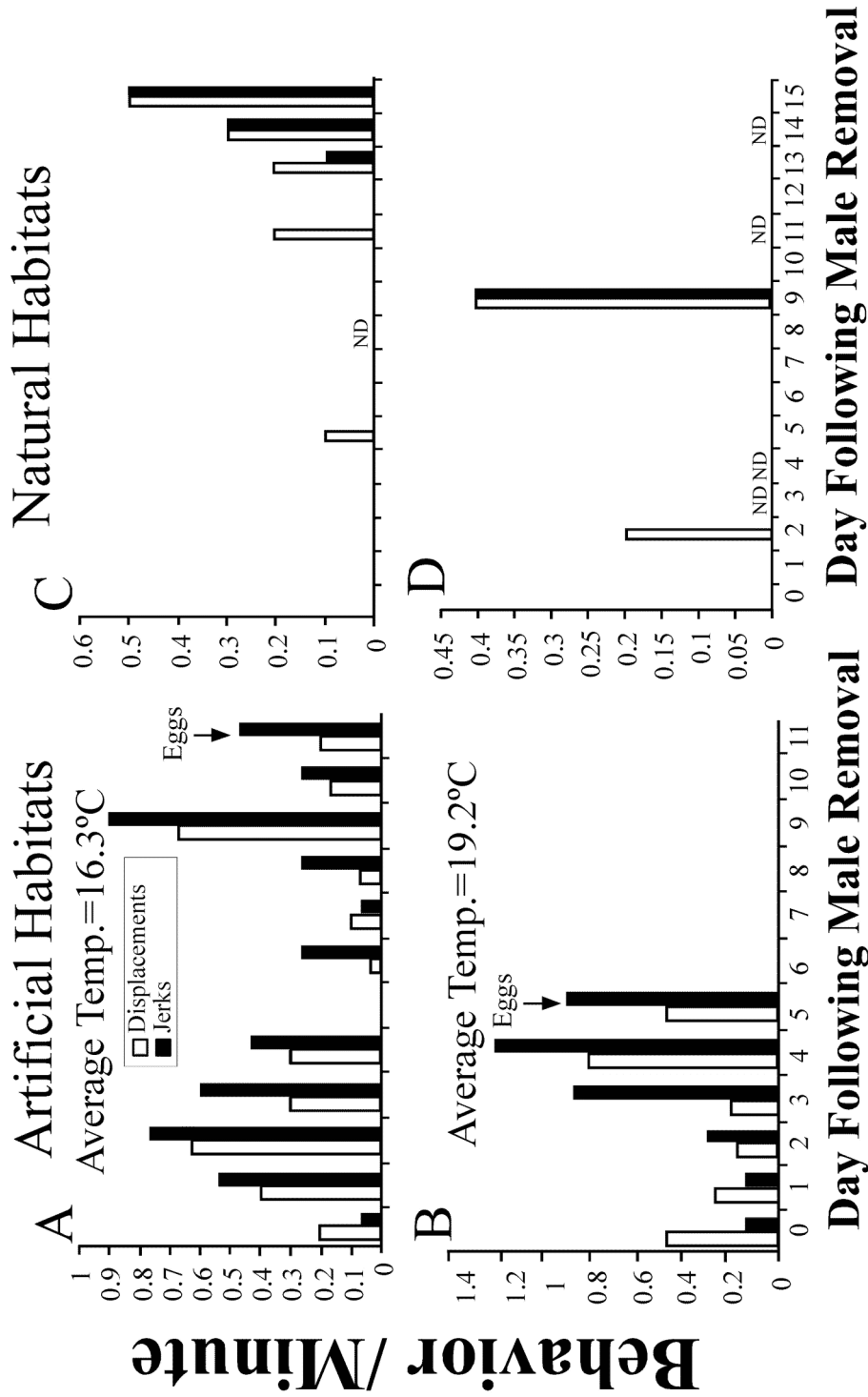


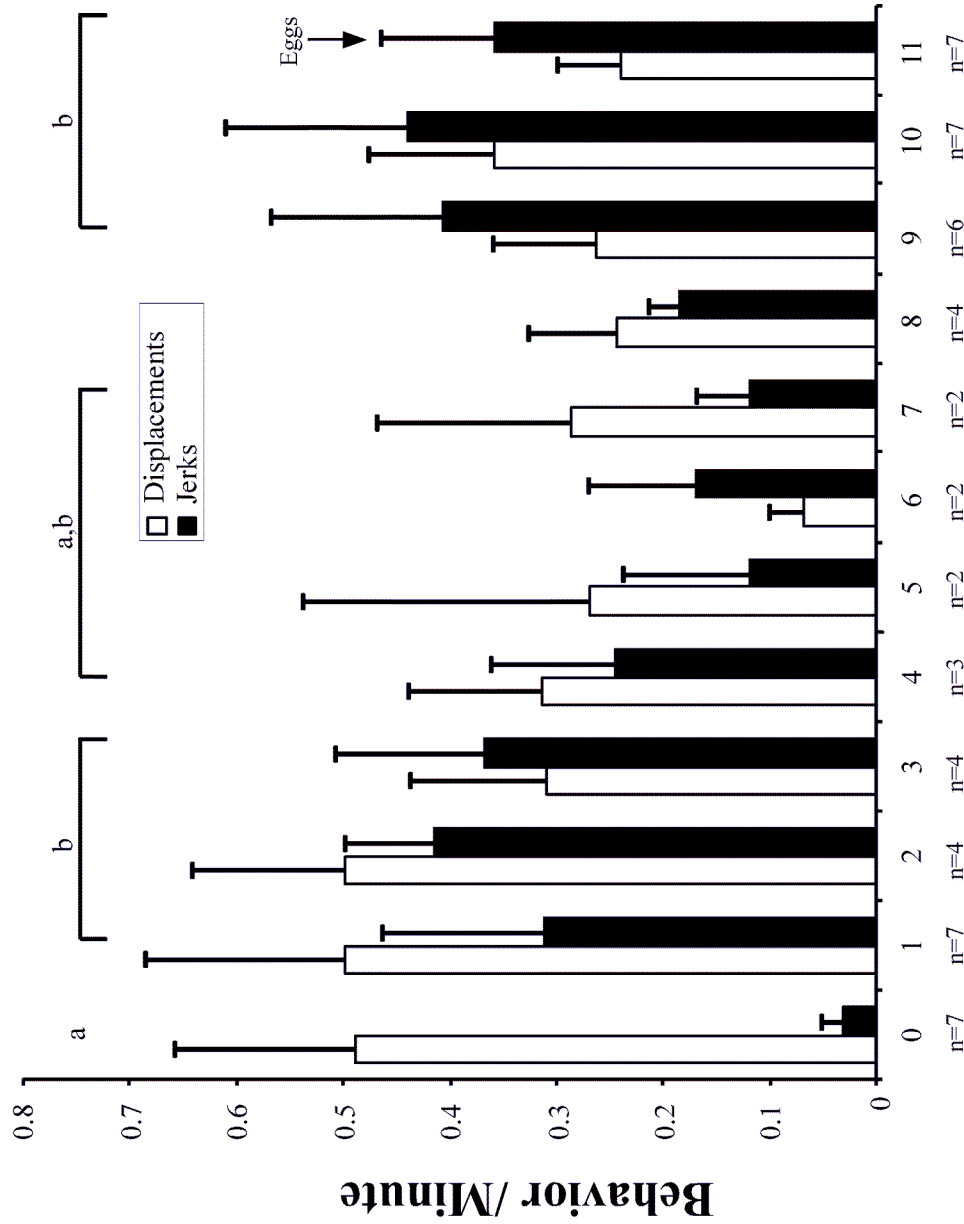
Figure 5: Four representative behavioral profiles of *L. dalli* focal fish after the male has been removed. Displacement and jerk behavior is shown for sex changers on artificial habitats (A and B) and focal fish on natural habitats (C and D). There is a slower rate of sex change in cooler temperatures early in the season (A) compared to the faster rate in warmer temperatures (B). Day 0 is the day of male removal. White bars represent displacements and black bars represent jerks. Arrows indicate when eggs were laid in the nest tube by non-focal fish. Note that the y-axis scales represent much smaller rates in C and D. ND=no data because fish was not seen.

contrast, the sex changing fish in profile two experienced 19.2°C and only took 5 days to fertilize eggs, requiring temperature correction for comparison between profiles (see methods).

Temperature-corrected displacements (Fig. 6) show two peaks, the first on day one following male removal ( $0.50 \pm 0.19$  displacements/minute) and the other on the day before fertilizing eggs as a male ( $0.36 \pm 0.12$  displacements/minute). These data could not be normalized through transformation procedures and the variation between groups was too high for repeated measures ANOVA to reveal a difference in displacement behavior over time.

Similarly, there were no statistical differences in displacements over time of focal females on natural habitats ( $n=6$ ), but three fish showed the same up, down, up behavior profile (Fig. 5C,D) at an even lower rate of displacements than those on artificial habitats. Fish in natural habitats exhibited both immigration to and emigration to and from the observed urchin group. This was insightful because a new male migrated in from a neighboring urchin to replace the male removed from the other three natural habitat groups, and instead of the behavioral profile shown by other focal fish, these females showed little to no displacements and displayed no jerking behavior following the immigration of a new male. The most frequent behavior in natural habitat focal fish was bites for food in the water column. Taking the average for each fish over the course of observations, 90.5% of the behavior was bites (0.87/min), 7.2% was displacements (0.069/min.), and 2.2% was jerks (0.022/min.).

Females, large or small, were never observed jerking before male removal, and following male removal only focal fish jerked. After the peak in displacements on the habitats, there was a peak of jerk behavior on day 2 after male removal ( $0.41 \pm 0.082$  jerks/minute; Fig. 6). During courtship, sex changers performed jerking and leading behavior toward the nesting tubes, and



### Day Following Male Removal (corrected for temperature)

Figure 6: Temperature-corrected displacement and jerk behavioral profiles over the course of sex change for all *L. dalli* sex changers on artificial habitats. White bars represent displacements and black bars represent jerks. Sample sizes varied across the days due to temperature correction, and different letters denote differences over time in averaged jerk behavior for clumped groups (see text for details). No significant difference was found in displacement behavior over time.

they entered the tubes repeatedly. Sex-changing fish increased jerking around the time eggs were laid, peaking the day before spawning (temperature-corrected day 10;  $0.44 \pm 0.17$  jerks/minute; Fig. 6). A repeated measures ANOVA revealed differences in jerking across time periods ( $F_{3,16}=4.05$ ,  $p=0.026$ ), with the day of male removal (day zero) being different from the average of days one to three ( $F_{1,16}=7.46$ ,  $p=0.015$ ) and days nine to eleven ( $F_{1,16}=10.44$ ,  $p=0.005$ ) but not days five to eight ( $F_{1,16}=2.72$ ,  $p=0.119$ ). The same pattern of jerking was seen in the focal females on natural habitats, though this was not statistically significant (Fig. 5C,D). As in the aquarium study, a leading behavior directing females to the spawning site was observed and only males or sex changers performed egg care. Along with egg care, some sex changers continued performing courtship (jerk) behavior toward other females in the group.

Counting only those groups maintained for more than three days after male removal and ignoring those fish missing due to urchin loss, 54 female fish were placed in the habitats. Fourteen fish were lost during the observation period, giving a survivorship rate of 74%. Of the missing fish, none were the largest, 9 (64%) were the medium sized fish ( $24.6 \pm 0.51$ mm), and 5 (36%) were the smallest fish ( $23.3 \pm 1.6$ mm). When only one fish was missing from a complete habitat (a compliment of three fish), 7 out of the 9 fish lost (78%) were the medium sized fish and only 2 (22%) were the smallest fish of the group.

#### *Histology: Intermediate between males and females*

After the experimental period, visual examination of all fish in the group showed only focal fish had male papilla morphology. The average length to width ratio of focal fish genital papilla was  $1.60 \pm 0.39$  with a range of 1.0 to 2.2. Focal fish papilla ratios were intermediate to

and significantly different from the papilla of females and males (females  $0.98 \pm 0.06$ , males  $3.48 \pm 1.07$ , Kruskal-Wallis test,  $\chi^2=16.7$ , Asymp. sig. $>0.001$ ).

Focal fish gonads had a mean cross-section area of  $3.7 \times 10^5 \pm 2.6 \times 10^5 \mu\text{m}^2$  with a range of  $9.0 \times 10^4$  to  $1.0 \times 10^6 \mu\text{m}^2$ . Cross-sections of female gonads averaged  $3.5 \times 10^5 \pm 3.0 \times 10^5 \mu\text{m}^2$  and males averaged  $1.3 \times 10^5 \pm 5.8 \times 10^4 \mu\text{m}^2$ .

While possessing very little testicular tissue, females displayed a large variability in ovarian tissue size (Fig. 7). Two fish had relatively minute gonads (mean cross-section =  $6.0 \times 10^4 \pm 2.2 \times 10^4 \mu\text{m}^2$ ). Three females with larger gonads (mean cross-section =  $5.5 \times 10^5 \pm 2.0 \times 10^5 \mu\text{m}^2$ ) were visibly gravid, with swollen and pink abdomens upon visual examination with a dissecting microscope. The two females with significantly smaller gonads had sunken abdomens and presumably had recently laid clutches. Omitting the two females with small gonads, the gonad sizes were significantly different between the males, females and focal fish. (Kruskal-Wallis test,  $\text{df}=2$ ,  $\chi^2=7.97$ , Asymp. sig.=0.019). Females had the largest gonads, males the smallest, and focal fish gonads were intermediate between males and females.

All focal fish gonads contained ovarian tissue and testicular tissue with spermatozoa. Percent testicular tissue in the gonad ranged widely from 0.8 to 65.4% with a mean of  $19.6 \pm 20.1\%$  for focal fish. In contrast, male fish gonads possessed a much more narrow range of  $95.4 \pm 7.5\%$  testicular tissue with spermatozoa. While the amount of testicular tissue comprising the male gonad varied, each gonad possessed little, or no ovarian tissue (Fig. 7). Females had  $1.04 \pm 0.89\%$  testicular tissue, in which 2 out of 5 possessed spermatozoa. The amount of testicular tissue in focal fish was significantly different from and intermediate to males and females (Kruskal-Wallis,  $\chi^2=17.8$ , Asymp. sig. $>0.001$ ).

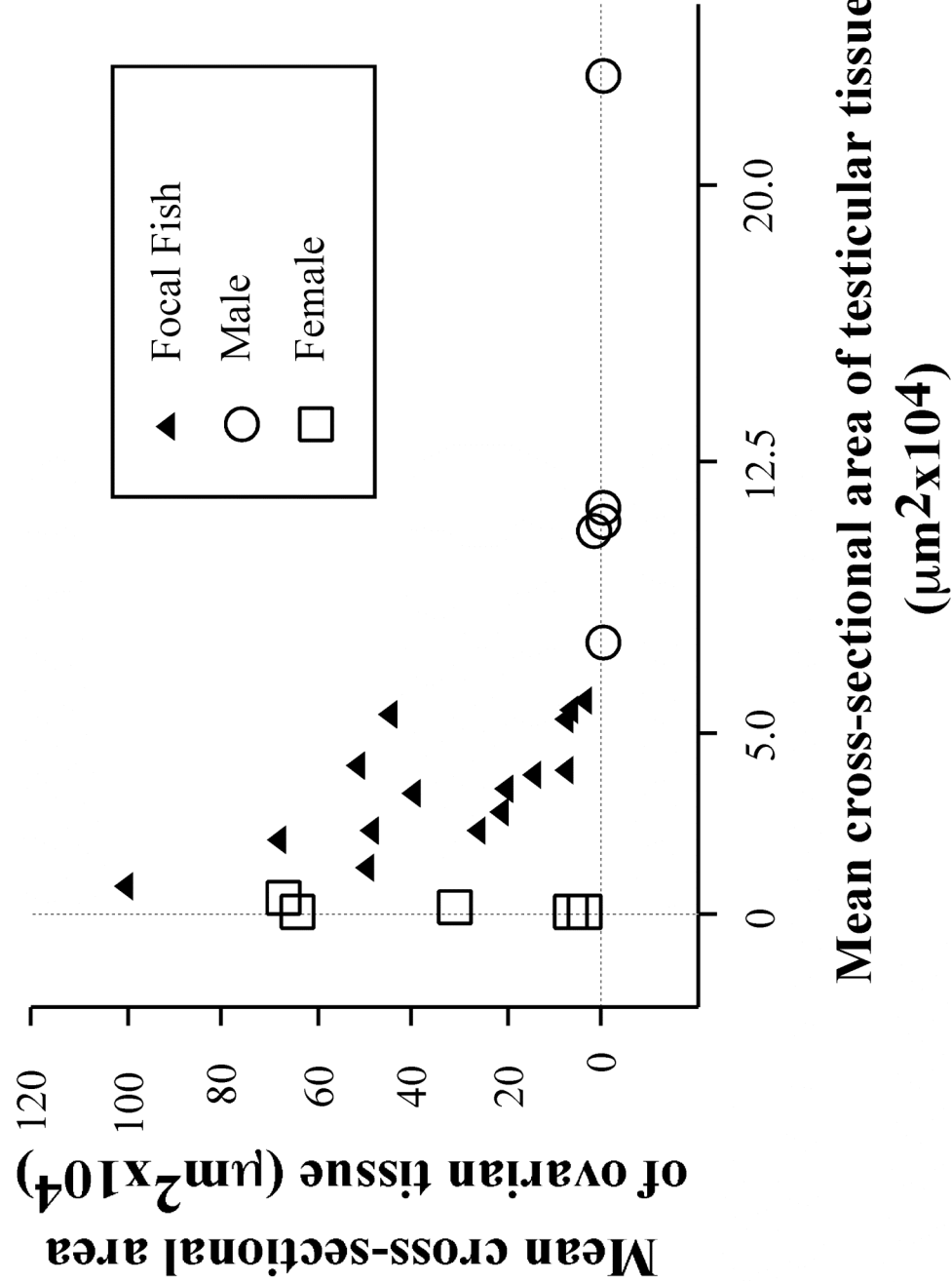


Figure 7: Scatterplot comparing mean ovarian and testicular allocations in transverse sections of *L. dalli* gonad in focal fish (closed triangles), males (open circles) and females (open squares).

In the gonads of focal fish, the amount of ovarian tissue decreased as the amount of testicular tissue increased. Therefore, the fish with the greatest ovarian tissue had the least testicular tissue and vice versa (Fig. 7). Analysis of tissues comparing the gonad from females to focal fish to males indicates that during protogynous sex change the rate of reduction of ovarian tissue is much less dramatic than testicular recruitment (Fig. 8). Mean ovarian cross-sections ranged from  $1.0 \times 10^6 \mu\text{m}^2$  in a focal fish having only 0.8 percent testicular tissue to no ovarian tissue in males with 100% testicular tissue. Conversely, testicular tissue ranged from a trace amount in females to  $2.2 \times 10^5 \mu\text{m}^2$  in male fish, which was almost an order of magnitude smaller than the largest ovarian section of gonad.

Comparing papilla length to width ratio to percent testicular tissue in the gonad across males, females and focal fish (Fig. 9) yielded a steeply sloped sigmoid curve ( $r^2=0.68$ ,  $F_{1,23}=50.9$ ,  $p<0.001$ ). Males, with the highest percentage of testicular tissue and most elongated papilla, and females, with the least amount of testicular tissue and blunt papilla, occupy the extreme ends of the curve. The focal fish display papilla and gonadal characteristics intermediate to males and females.

All focal fish possessed an AGS, averaging  $5.8 \times 10^4 \pm 3.2 \times 10^4 \mu\text{m}^2$  in cross-sectional area (range:  $1.4 \times 10^4$  to  $1.2 \times 10^5 \mu\text{m}^2$ ). The mean AGS area of males was significantly larger than those of focal fish ( $2.7 \times 10^5 \pm 1.5 \times 10^5 \mu\text{m}^2$  vs.  $5.8 \times 10^4 \pm 3.2 \times 10^4 \mu\text{m}^2$ , Mann-Whitney U-test,  $U=0$ ,  $p<0.01$ ,  $n_1=15$ ,  $n_2=5$ ). No female had an AGS.

In focal fish and males, the mean AGS area correlated with percent testicular tissue in the gonad (Fig. 10;  $r^2=0.60$ ,  $F_{1,18}=27.02$ ,  $p<0.001$ ). In fish with a small AGS, little testicular tissue and blunt papilla, the AGS is initially restricted to the caudal portion of the gonad close to the

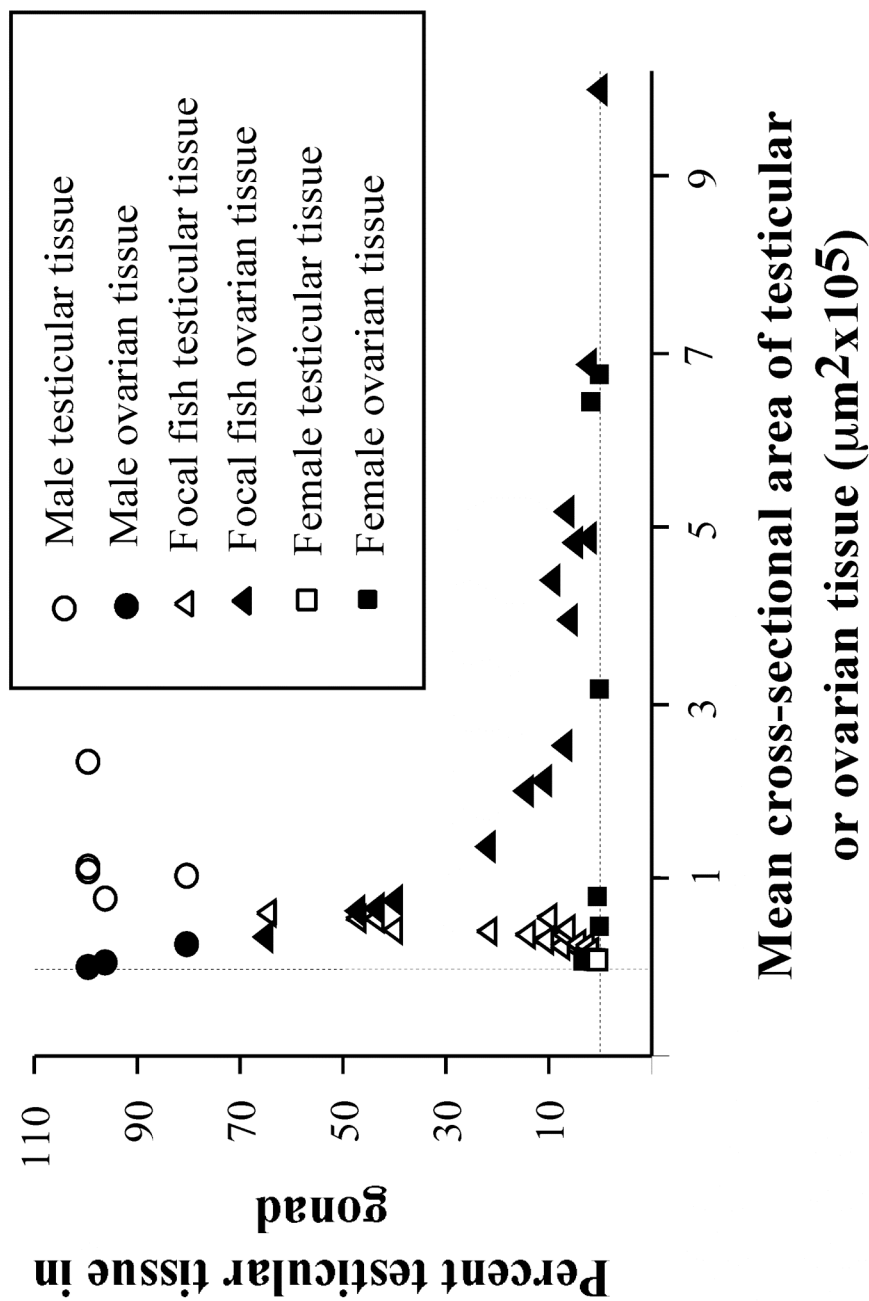


Figure 8: Scatterplot comparing percent testicular allocation in *L. dalli* gonad (testicular tissue size/ gonad size  $\times 100$ ) to mean transverse cross-section size of testicular tissue (open shapes) and ovarian tissue (closed shapes) in males (circles), focal fish (triangles) and females (squares).



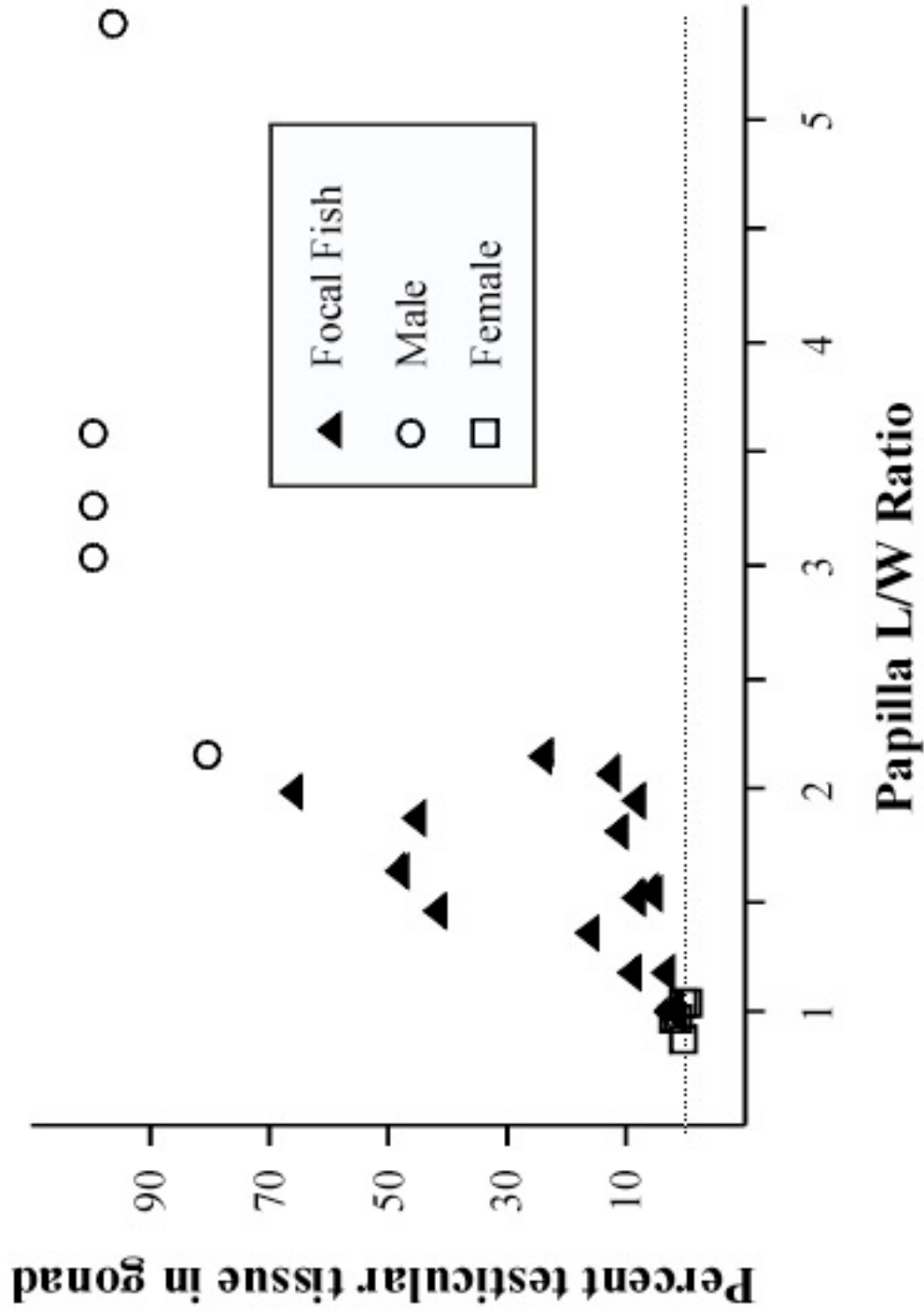


Figure 9: Scatterplot comparing percent testicular tissue allocation in *L. dalli* gonad (testicular tissue size/ gonad size x 100) to mean transverse cross-section size of testicular tissue (open shapes) and ovarian tissue (closed shapes) in males (circles), focal fish (triangles) and females (squares).

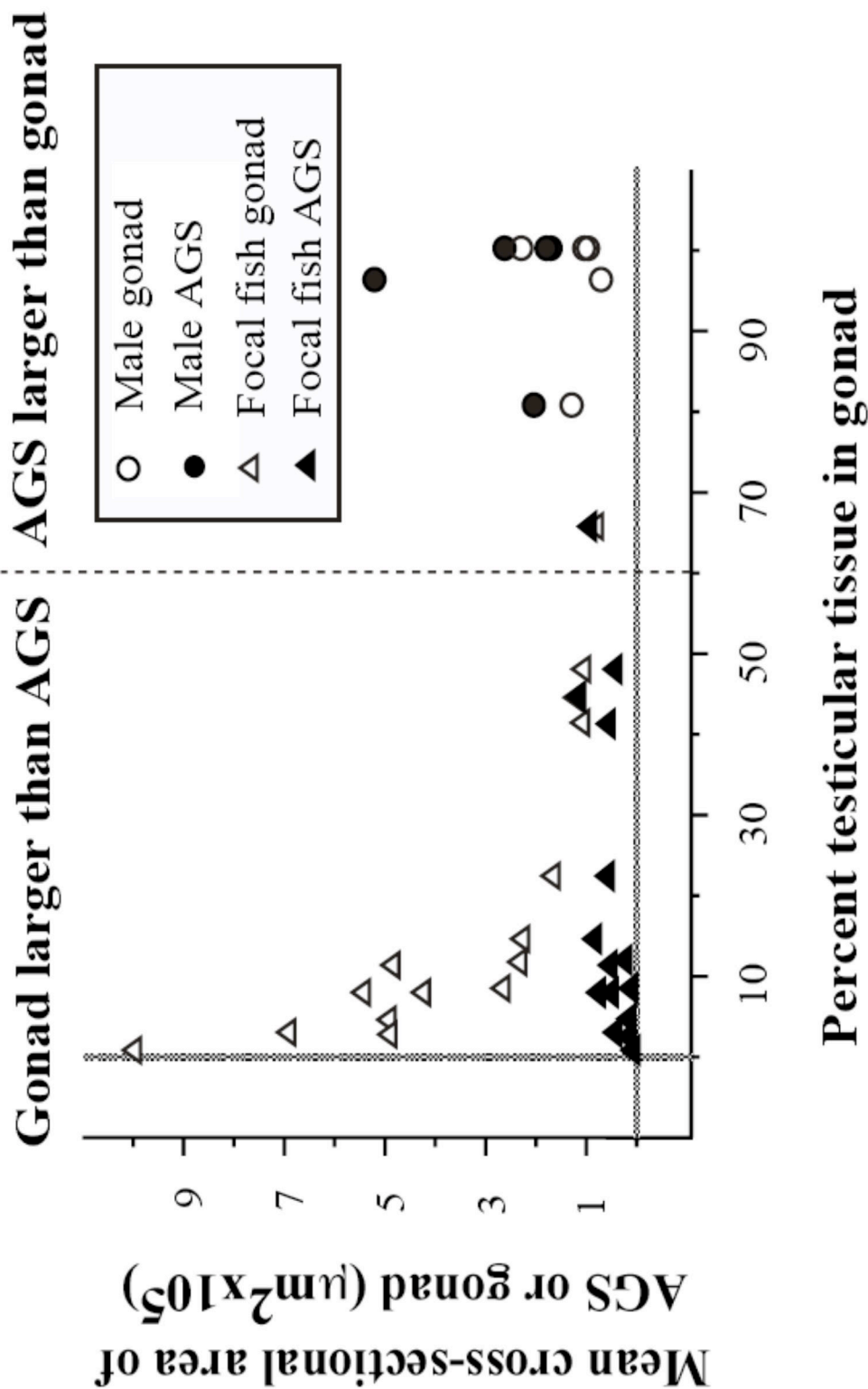


Figure 10: Scatterplot comparing percent testicular tissue allocation in *L. dalli* gonad (testicular tissue size/ gonad size x100) to mean transverse cross-sectional area of gonad (open shapes) or AGS (closed shapes) of focal fish (triangles) and males (circles).

vent. As morphology becomes increasingly male, the organ enlarges and expands anteriorly along the length of the gonad.

Analysis of the tissues from focal fish to males indicates that gonad size rapidly decreased as the percentage of testicular tissue increased (Fig. 10). Simultaneously, AGS size increased with the percentage of testicular tissue in the gonad. In fish whose gonad was composed of more than 60% testicular tissue, the mean cross-sectional area of the AGS was larger than that of the gonad. The AGS of six focal fish had a category zero sperm count, five had category one, and four had category two sperm counts (see methods; category zero:  $2.8 \times 10^4 \pm 1.4 \times 10^4 \mu\text{m}^2$ , category one:  $7.2 \times 10^4 \pm 1.5 \times 10^4 \mu\text{m}^2$ , category two:  $8.5 \times 10^4 \pm 3.6 \times 10^4 \mu\text{m}^2$ , Kruskal-Wallis test,  $\text{df}=2$ ,  $\chi^2=13.6$ , Asymp. sig.=0.001). In comparison to focal females, males had more sperm aggregations in the AGS.

Focal fish AGS had numerous, well-defined partitions between tubules spanning the width of the cross-section. In comparison, cross-sections of male AGS had far fewer interior walls. Sections of male fish AGS with mean wall thickness of less than  $10 \mu\text{m}$  showed signs of these walls bursting (partial segments of walls not spanning the width of the organ as in focal fish). Male AGS morphology more closely resembled a single, continuous organ than the series of sub-compartments seen in the focal fish. The largest AGS of a male fish contained only one measurable internal wall. Due to insufficient sample size, the wall thickness data from this fish was not used in calculations.

Mean AGS wall thickness of focal fish ranged from  $39$  to  $67 \mu\text{m}$ . Focal fish AGS walls were significantly thicker than those of males ( $51.2 \pm 9.2$  vs.  $16.7 \pm 7.2 \mu\text{m}$ , Mann-Whitney U-test,  $U=0$ ,  $p<0.01$ ,  $n_1=15$ ,  $n_2=5$ ). In focal fish and males, mean AGS wall thickness negatively

correlated with AGS cross-sectional area and the genital papilla ratio (Fig. 11;  $r^2=0.72$  and  $r^2=0.64$ , respectively).

Focal fish that fertilized eggs and those not fertilizing eggs showed no significant difference in papilla ratio ( $1.69 \pm 0.31$  vs.  $1.49 \pm 0.47$ , Mann-Whitney U-test,  $U=20$ ,  $p=0.354$ ,  $n_1=7$ ,  $n_2=8$ ), amount of testicular tissue ( $18.5 \pm 17.4\%$  vs.  $20.8 \pm 24.3\%$ , Mann-Whitney U-test,  $U=20$ ,  $p=0.354$ ,  $n_1=7$ ,  $n_2=8$ ), average AGS cross-sectional area ( $6.8 \times 10^4 \pm 2.9 \times 10^4 \mu\text{m}^2$  vs.  $4.7 \times 10^4 \pm 3.4 \times 10^4 \mu\text{m}^2$ , Mann-Whitney U-test,  $U=17$ ,  $p=0.10$ ,  $n_1=7$ ,  $n_2=8$ ), or sperm count categories ( $1.13 \pm 0.83$  vs.  $0.57 \pm 0.79$ , t-test, equal variances not assumed,  $df=12.9$ ,  $t=1.3$ , 2-tailed sig.=0.21).

#### *Steroids: Linking physiology to morphology and behavior*

Free and conjugated (sulfate and glucuronide) forms of cortisol, 11-KT and E2 were detected in all water samples from focal fish. The cortisol levels were higher than 11-KT or E2 levels (free cortisol:  $426.9 \pm 103.3$  pg/sample, ranging from 71.1-1427.4 pg/sample; total cortisol:  $1576.6 \pm 465.4$  pg/sample, ranging from 159.8-5932.6 pg/sample; sulfate conjugated cortisol:  $957.3 \pm 373.0$  pg/sample, ranging from 63.7-5340.0 pg/sample; glucuronide cortisol:  $192.4 \pm 71.1$  pg/sample, ranging from 18.3-1050.1 pg/sample). Total 11-KT (range 77.3-310.1 pg/sample, mean  $147.2 \pm 55.0$ ) was significantly greater than E2 (range 15.0-120.6 pg/sample, mean  $50.5 \pm 29.8$ , paired t-test,  $p<0.001$ ) with an 11-KT/E2 ratio of  $4.9 \pm 5.1$ . The amount of conjugated 11-KT per sample was greater than free 11-KT ( $107 \pm 51.2$  vs.  $39.7 \pm 22.5$  pg/sample, paired t-test,  $p>0.001$ ). The amount of sulfate conjugated 11-KT in each sample was greater than glucuronide conjugated 11-KT ( $70.8 \pm 28.9$  vs.  $36.8 \pm 26.7$  pg/sample, paired t-test,

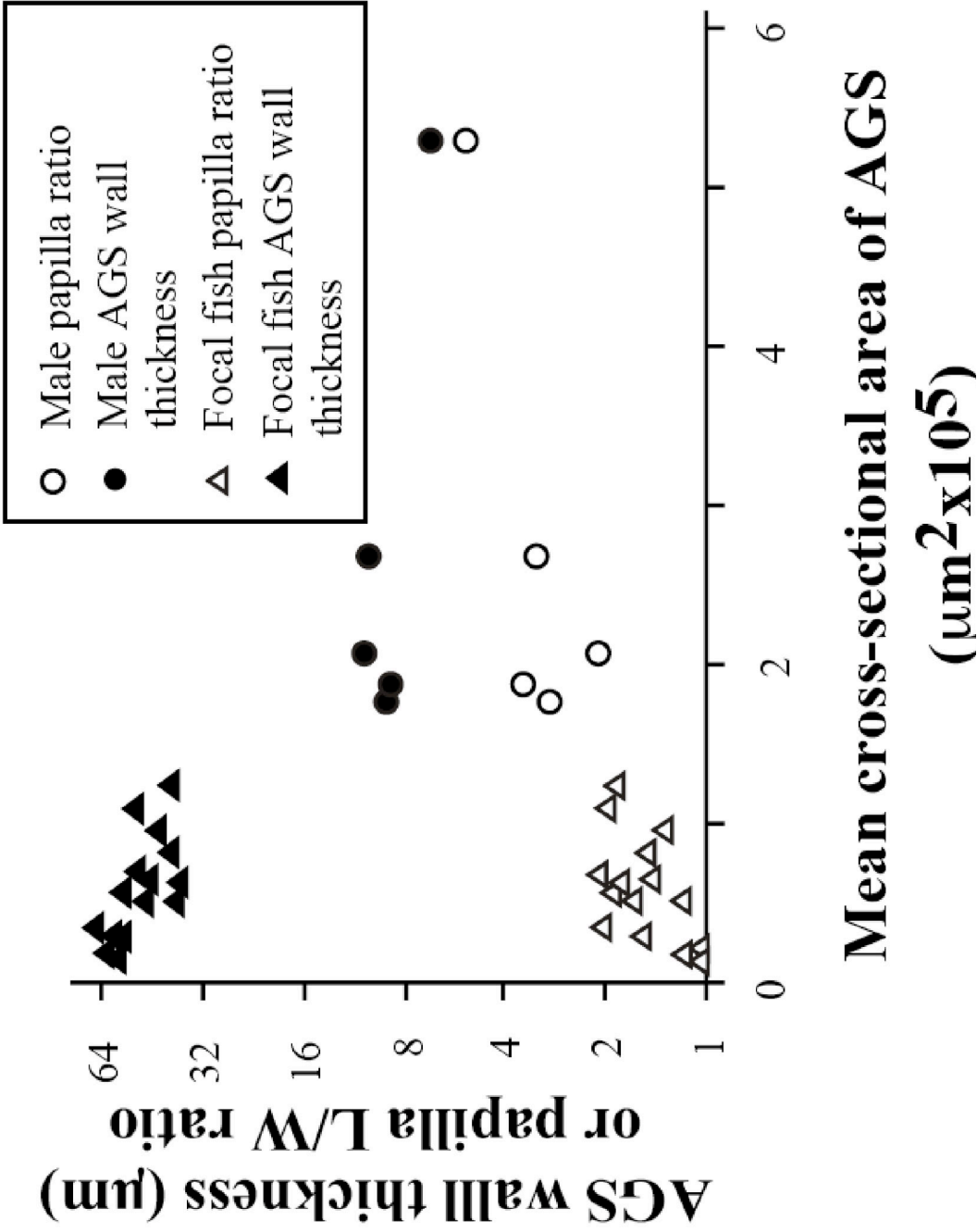


Figure 11: Scatterplot comparing *L. dalli* AGS interior wall thickness (closed shapes) and genital papilla length to width ratio (open shapes) to the mean transverse cross-sectional area of AGS in focal fish (triangles) and males (circles).

$p < 0.001$ ).

Total, conjugated, and free 11-KT concentrations did not significantly correlate with any histological measurements. However, teleosts have been shown to have a morning (AM) androgen peak, with basal urinary androgens highest in the morning and continually declining throughout the day (Oliveira, 2001e). Because only a single sample was taken from each fish at the time of removal, a decline in androgens over the day could not be determined. However, mean 11-KT levels in samples from afternoon (PM) fish had less, though not significantly less, 11-KT than AM fish ( $45.9 \pm 9.3$  vs.  $35.6 \pm 7.6$  pg/sample; ANOVA,  $F_{1,13}=0.739$ ,  $p=0.41$ ). Controlling for time of sampling by just examining morning samples uncovered several interesting relationships between steroid levels and both behavior and reproductive anatomy. Free 11-KT from morning samples significantly and positively correlated with percent testicular tissue ( $r^2=0.791$ , ANOVA,  $F_{1,4}=15.11$ ,  $p=0.018$ ), average size of the AGS ( $r^2=0.837$ , ANOVA,  $F_{1,4}=20.60$ ,  $p=0.011$ ), and displacement behavior on the last day before sampling ( $r^2=0.786$ , ANOVA,  $F_{1,4}=14.68$ ,  $p=0.019$ ), but not genital papilla length to width ratio ( $r^2=0.022$ , ANOVA,  $F_{1,4}=0.091$ ,  $p=0.778$ ; Fig. 12). The 11-KT/E2 ratio also correlated with some of these traits, but did not account for as much of the variance as 11-KT alone.

## Discussion

### *Behavior: Lower rates, but similar patterns*

Much of the behavior that occurred in the field was similar to the description of Reavis and Grober (1999). Corresponding to aquarium-based results, sex change proceeded faster in the

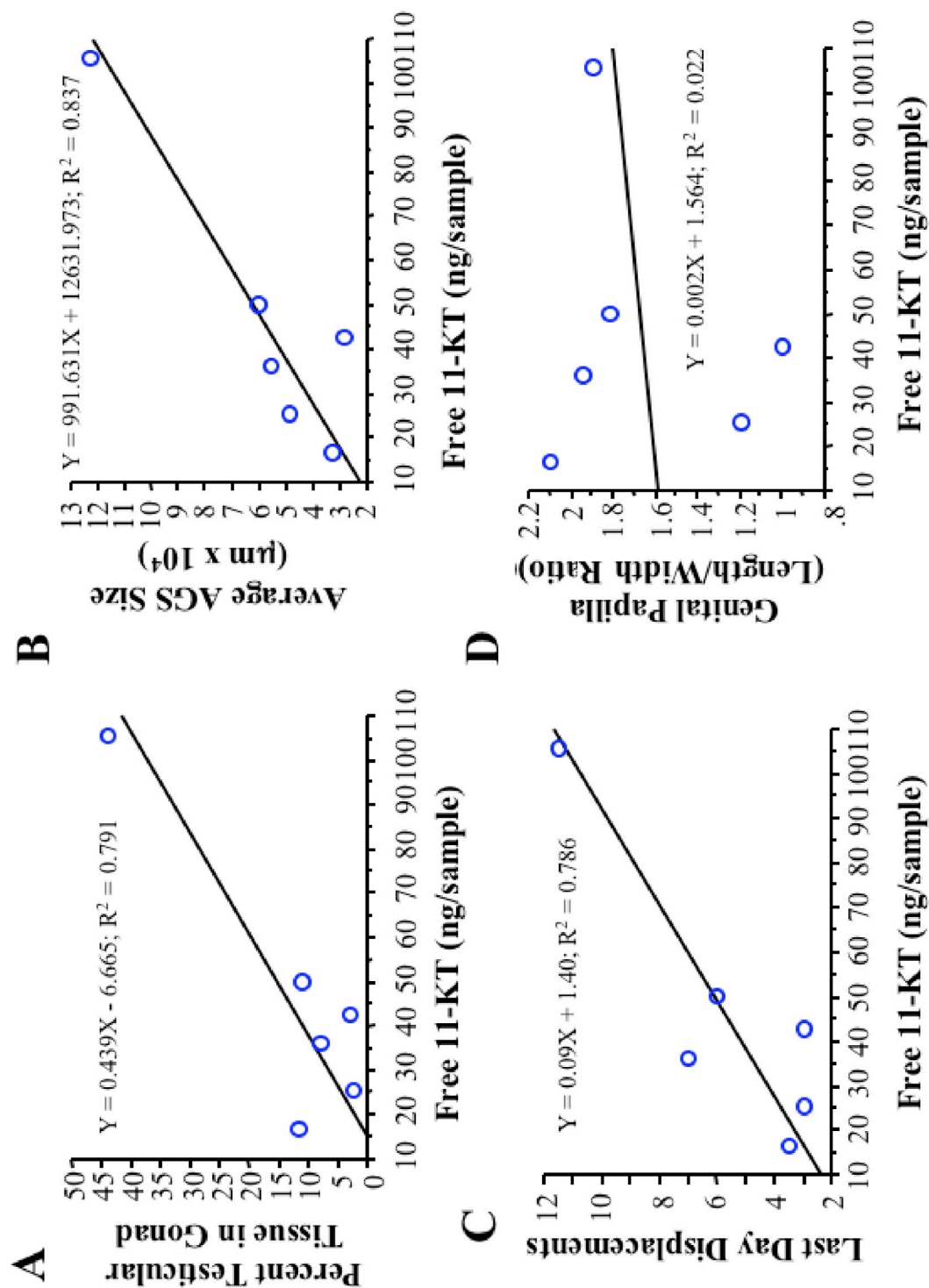


Figure 12: Free 11-ketotestosterone (11-KT) samples taken in the morning correlate with percent testicular tissue in the gonad (A), average accessory gonadal structure size (B), and last day displacements (C), but not genital papilla ratio (D) in *L. dalli*.

summer than the spring. In May, the longest time to change sex in the field was 10.5 days, which is comparable with Reavis and Grober's finding of  $9.0 \pm 2.2$  days to change sex in the spring. In late June, the fastest sex change in the field was 5 days, again similar to the  $5.5 \pm 2.3$  days observed in aquaria. The faster sex change may be a result of physiologic changes taking place more rapidly within the sex changer or the females in the group having a faster rate of egg laying so that the changer has eggs to fertilize more quickly. While Reavis and Grober (1999) found the largest female fish was marginally more likely to change sex, in this study only the largest females fertilized eggs as a male, and only one group had the second largest fish as a focal fish (dominant over the largest fish).

The sample behavioral profiles (Fig. 5A,B) show a common pattern of behavior, despite variability in the frequency of behavior and time needed to change sex. Because of the variability, even when behavior profiles from all sex changers were corrected for temperature (Fig. 6), large standard errors were obtained. Despite this variance, two increases in displacements correlating with post-male removal and later courtship as a male can be seen, as in Reavis and Grober (1999). The behavioral observations from groups with males removed on natural habitats in the field show similar behavior profiles (Fig. 5C,D), and these are distinctly different from the profiles seen in groups with a male present, suggesting that the behavioral profile described in aquaria is similar to what occurs in both artificial and natural habitats in the field.

Similar to Reavis and Grober (1999), the temporal sequence of sex change behavior in the field consisted of an active dominance phase, a quiescent phase and an active courtship phase (Figs. 3,5,6). Although the behavioral pattern was similar between the lab and field, behavioral rates were lower under natural conditions. Displacements first peaked in the field at  $0.50 \pm 0.19$



per minute, which is less than the  $2.18 \pm 1.86$  recorded in laboratory studies. As in the lab, the initial peak in displacements occurred in less than three days. In the field and laboratory, displacements peaked a second time in association with spawning as a male. During this peak, the field rate of  $0.36 \pm 0.12$  displacements per minute was less than the laboratory rate of  $1.37 \pm 1.71$ .

Concomitant with the second displacement peak, sex changers displayed a peak in jerking (Fig. 5). Peak jerking rates on artificial habitats in the field,  $0.44 \pm 0.17$  jerks/minute, were lower than laboratory-recorded rates,  $0.73 \pm 0.34$  jerks/minute. In addition, the early peak in jerks followed the displacement peak, as in the model, and with this lag time, the jerks on the day of male removal were significantly lower than the jerk behavior during the rest of dominance (days 1-3) and courtship (days 9-11) phases, but not the quiescent phase (days 5-8) (Figs. 3,5). Following male removal, only focal fish performed jerks. Thus, jerking behavior was a strong indicator of initiation of sex change and ultimately the new "maleness" of the focal fish.

Many variables could explain lower rates of behavior in the field during sex change. Variability of individual fish, their manipulated social groups, the interactions of individuals with their environments, and the experimental construct created natural and artificial stressors on the groups. However, four factors seemed most influential. First, field observations of the focal fish indicate a large portion of active *L. dalli* behavior is foraging (90.5% for this study, with similar rates to St. Mary, 1994a; Steele, 1996; Steele, 1998). Instead of feeding to satiation on flake food twice a day, fish in the habitats fed on plankton continually through the day, reducing time for intraspecific interactions. Second, intruders into the habitat, most notably blackeye gobies, *Rhinogobiops* (= *Coryphopterus*) *nicholsii*, added social complexity and interspecific interactions that also reduced the time available for intraspecific behavior. Third, some of the groups in the

aquarium-based studies had one or two more females per group, so my focal fish had an average of one less fish to interact with (Reavis and Grober, 1999). Fourth, kelp bass, *Paralabrax clathratus*, sand bass, *P. nebulifer*, and other predators restricted the fish from interacting as often as they would in the aquaria without predators (Steele, 1996; Steele, 1998). Predation also resulted in smaller groups and fewer potential mates.

Predation had an impact on the groups that can be compared to other field studies. An overall survivorship rate of 74% was consistent with the 80% recorded in field experiments by St. Mary (1994a), and higher than the 10-48% survivorship reported by Steele (1996; 1998) in rubble piles without urchins. However, *L. dalli* survivorship was not size-independent as previously recorded (St. Mary, 1994a). Medium sized fish were more likely to be missing. This discrepancy may be due to differences in habitat, but it also raises the question of possible marginalization of the next smallest fish during the sex change process. If the sex changer begins inhibiting sex change in other females, the inhibition may be focused on probable competitors for dominance and sex change. The second largest fish could be marginalized from the group and exposed to higher predation as a result of this higher aggression from the sex changer. However, this cannot be conclusively determined from the data collected. Study of the distribution of displacements toward conspecifics of differing sizes and the possibility of increased predation is needed.

#### *Histology: Transitional, but Functionally Male*

Histology of focal fish showed a spectrum of morphologies, allowing for the analysis of the interrelationship of the gonad, the papilla and the AGS as related to protogynous sex change.

As the sex-typical behavior of a female fish changes, histology shows that the gonad is restructured and masculinized, the papilla elongates and narrows, the AGS forms and expands, sperm production increases, and sperm aggregations are sequestered in the AGS. My results are consistent with observations that during protogynous sex change, the AGS develops in association with the newly formed testis (Cole and Robertson, 1988; Cole and Shapiro, 1990).

Females showed variation in gonad size, but all possessed mostly ovarian tissue and minimal amounts of testicular tissue. Gonads of males had little to no ovarian tissue and a range of testicular tissue sizes. Male gonad size is small relative to female gonad size (Fig. 10). This implies 1) a reduction of gonad size during sex change and 2) that small testes produce sufficient sperm for fertilization of available eggs. Only fish undergoing sex change had gonads with a relatively equal amount of testicular and ovarian tissue.

In terms of protogyny, “transitional” implies a fluctuation between function as a female and function as a male, but individual *L. dalli* possessing both testicular and ovarian tissues exclusively display only male or only female behavior and appear to reproduce only as that sex (St. Mary, 1994b; Reavis and Grober, 1999). Sex changing fish in this study functioned as a male, and therefore should not be considered sexually transitional. Histology showed that fish with very little testicular tissue and a developing AGS were able to function as a male. Comparing the “new male” sex changers with the representative males, a difference can be observed between “functional” and “optimal” males. Male fish as defined by St. Mary (1993) can have between 5 and 100% testicular tissue, but those with large AGS, fully elongated papilla and purely testicular gonad appear to have optimized their male function.

Fishelson (1991), in a review of gobiid AGS morphology, found that as spermatogenic tissues proliferate, the AGS rapidly fills with mucus secretion and sperm. The initially thick

cuboid cells of the internal epithelium stretch, becoming elongated and flat. The internal walls were absent in developed males. These observations are consistent with the AGS morphology of *L. dalli* and the decreased AGS wall thickness of males compared to sex changing fish observed in this study (Fig. 11). The decreased number of chambers and the thinning of internal walls over time suggest that the AGS fills as it develops and the inner walls are stretched and burst.

The hypothesized functions of the AGS are varied (reviewed in Barni *et al.*, 2001; Cole and Hoese, 2001), but the AGS secretions embed the sperm and can facilitate adherence to the nesting site. As the AGS incorporates sperm in the appropriate delivery medium, the elongation of the papilla may increase its effectiveness in delivering sperm trails to the nest site. If male fish use sperm trails for asynchronous fertilization, then a larger AGS could produce larger, longer-lasting sperm trails important in sperm competition (Marconato *et al.*, 1996; Scaggiante *et al.*, 1999). This explains why an “optimal” male has an AGS larger than its testis.

The generally accepted paradigm in fishes and other vertebrates is to use genitalia and/or gonadal histology to assign sex (*e.g.* Cole and Shapiro, 1990). However, in my study, one sex changer fertilized eggs as a male but possessed only 3% testicular tissue and had a papilla ratio of 1.2, both below the accepted norms of male morphology for *L. dalli* (greater than 5% testicular tissue and having a papilla length to width ratio of greater than 1.4; Carlisle *et al.*, 2000). Compared with three other fish that had a similar testicular tissue allocation but did not fertilize eggs, this successful sex changer had a substantially larger AGS (average cross section  $4.9 \times 10^4$  vs.  $3.3 \times 10^4 \mu\text{m}^2$ ). Another sex changer with a similar sized AGS had 48% testicular tissue. The fertilization success of both of these individuals, despite their widely varying percentage of testicular tissue, suggests the importance of the AGS in male reproduction and that relative masculinity in *L. dalli* is not best quantified by percent testicular tissue or genital papilla,

but rather by the size and functionality of the AGS. Indeed, the male genital papilla can, in some instances, be a poor indicator of gonadal function (St. Mary, 1993), and female *L. dalli* can possess spermatozoa, but they are unlikely to function as males without effective sperm delivery through organs such as the AGS and a male-like genital papilla.

According to Sadovy and Shapiro (1987), definitive proof of sequential hermaphroditism requires the production of sex-changing individuals experimentally, using non-hormonal techniques, in conditions that closely resemble surroundings that may occur in nature. Analysis of focal fish in this study confirmed sequential hermaphroditic sex change in my experimental groups. All focal fish were behaviorally dominant, displayed jerking behavior, and, unlike females, possessed an AGS. However, histological analysis did not find a significant difference between fertilizers and non-fertilizers. This may be a result of the assay. In this study, a conservative measure of sex change, fertilization of eggs, was used as a definitive functional display of maleness. Within two weeks, focal fish fertilized eggs as a male in 9/15 (60%) groups, as compared to 13/17 (77%) in the lab. Some focal fish may have changed sex and possessed the ability to fertilize eggs but lacked a gravid female with which to mate. Previous studies have shown *L. dalli* has a three-week interclutch interval (Behrents, 1983; St. Mary, 1994b). In my experiment, only two females were available to each sex changer and predation occasionally reduced this number to only one, while some groups in the aquarium studies had more than three females in a group (Reavis and Grober, 1999).

*Steroids: Linking Physiology with Behavior and Morphology*

Cortisol may be high due to the energy demands of changing sex and/or higher levels of jerk and displacement behavior. The 11-KT/E2 ratio in the *L. dalli* focal fish varied widely ( $4.85 \pm 5.1$ ) but was similar to the ratio of approximately 4:1 found by Kroon and Liley (2000) for male *Rhinogobiops nicholsii*, another protogynous goby. 11-KT levels are particularly interesting because 11-KT is the most potent fish androgen (Borg, 1994). Lab female *L. dalli* implanted for 3-5 days with 11-KT developed an elongated male-typical papilla (Carlisle *et al.*, 2000), enlarged testicular tissue, regressed ovarian tissue, and a male-typical, sperm-sequestering AGS (Carlisle, 2001). The lack of a positive correlation between 11-KT levels and genital papilla morphology in my study is not consistent with Carlisle *et al.* (2000). This may be because 11-KT promotes the lengthening of genital papillae at very high levels, such as those with the implants, but not at endogenous levels. My results for internal morphology were in agreement with expectations from Carlisle (2001), showing that alterations in the percent testicular tissue and AGS are highly correlated with morning levels of endogenous free 11-KT and reinforcing the idea that these sex characteristics may be responding to a common androgenic mechanism during sex change. These results are also consistent with reports of 11-KT resulting in testicular tissue induction in other teleosts such as the goldfish, *Carassius auratus* (Kobayashi *et al.*, 1991), and 11-KT stimulating spermatogenesis in the Japanese eel, *Anguilla japonica* (Miura *et al.*, 1991). In this study, it appears testicular tissue was recruited at a much faster rate than ovarian tissue was reduced (Fig. 8). These results support findings from aquarium studies investigating gonad changes over the course of sex change (Black, Nowak, Moore, and Grober, unpublished data). In another sex changing species, the protogynous bluehead wrasse, *Thalassoma bifasciatum*, Kramer *et al.*

(1988) found a decrease in ovarian tissue, but no induction of testicular tissue following treatment with the 11-KT precursor, testosterone. This may be a result of a lack of conversion of testosterone to 11-KT. Elevated 11-KT is associated with protogynous sex change in *Sparisoma viride* (Cardwell and Liley, 1991b), *Epinephelus merra* (Bhandari *et al.*, 2003a), *T. duperrey* (Nakamura *et al.*, 1989), and *R. nicholsii*, although in *R. nicholsii* there was a great deal of overlap in 11-KT levels between males and females (Kroon and Liley, 2000). Further, 11-KT seemed more associated with territoriality in *S. viride* (Cardwell and Liley, 1991a), *E. merra* compared for 11-KT had been put into stages of sex change based on their gonadal state rather than when they had begun to change sex, and female *T. duperrey* and males that had just changed from female had essentially the same 11-KT levels, even though established male 11-KT levels were higher (Nakamura *et al.*, 1989). Higher 11-KT was also associated with males before protandrous (male to female) sex change in the anemone fish, *Amphiprion melanopus* (Godwin and Thomas, 1993), dominant male fish in all male groups of *Oreochromis mossambicus* (Oliveira *et al.*, 1996), male secondary sex characteristics in *Parablennius* and *S. pavo* (Oliveira *et al.*, 2001a,b,c), and courting male morphs in species with male dimorphism (Brantley *et al.*, 1993).

In addition to the morphological correlation, this study showed a behavioral correlation to free 11-KT. The positive correlation between morning free 11-KT and the rate of displacement just before sacrifice suggests that either the behavioral interactions of the fish influence the steroid levels, as seen in the challenge hypothesis, or that higher 11-KT levels influenced the behavior of the focal fish (Wingfield *et al.*, 1990; Oliveira *et al.*, 2001e).

In conclusion, I have established that sex change in the bluebanded goby can be induced under natural conditions by removal of the dominant male from small, isolated social groups.

The dynamics of behavioral sex change in the field are quite similar to those described under laboratory conditions. The absolute amount of social behavior is reduced in the field, probably due to the increase in time spent foraging and avoiding predators. Temperature had a significant effect on the time required to change sex with complete sex change taking almost twice as long early in the summer relative to the warmer late summer months. Histological analysis of sex changing fish revealed gonads, genital papillae, and accessory gonadal structures with morphology that is transitional to that of representative females and males. Fish with significant testicular development, accessory gonadal structure growth, and more frequent aggressive behavior exhibited higher levels of free 11-KT in the morning, linking endogenous levels of steroids to behavioral and morphological changes. These results demonstrate that sex change in this species can be effectively studied under field conditions and this can provide researchers with a unique opportunity to understand the degree to which naturally occurring variation within social groups, between species (*e.g.* predator/prey relationships), and in the abiotic environment can regulate sexual function and reproductive success.



### CHAPTER 3: SOCIALLY-REGULATED SEXUAL DIFFERENCES IN PREOPTIC ISOTOCIN-LIKE IMMUNOREACTIVE CELLS

(figures and text modified with permission from a published version: Black, M.P., Reavis, R.H., and Grober, M.S. 2004. Socially induced sex change regulates forebrain isotocin in *Lythrypnus dalli*. *Neuroreport*, 15(1):185-189.)

Author roles: M.P. Black, R.H. Reavis, and M.S. Grober designed the experiment and collected and analyzed the data. M.P. Black and M.S. Grober wrote the manuscript.

#### Summary

The neurohypophyseal peptides are evolutionarily conserved and their expression can be socially modulated. My question was: what effect will socially induced sex change have on forebrain isotocin, an oxytocin homologue? Males were removed from social groups to induce dominant females to change sex and become males in *Lythrypnus dalli*. Fish in the late stages of sex change had fewer forebrain isotocin-like immunoreactive (IST-ir) cells than early stage and unchanged females. When groups were consolidated into unequivocal males (control males and sex-changed new males) and unequivocal females (fish prior to courtship as a male), females had significantly more IST-ir cells than control males and recently sex-changed fish. This is the first study demonstrating the social regulation of forebrain isotocin.

## Introduction

Social interactions can regulate the brain and reproduction. For example, social signals can cause an African cichlid fish to change from nonreproductive to reproductive status and these same social signals result in increases in the size of gonadotropin-releasing hormone cells in the brain (Francis *et al.*, 1993). The neuropeptide oxytocin is also modulated by social interactions. Defeated male rats release more oxytocin within the hypothalamus than those that are not defeated (Engelmann *et al.*, 1999). Social dominance also alters the behavioral response of female Syrian hamsters to centrally administered oxytocin (Harmon *et al.*, 2002).

Oxytocin has been implicated in the regulation of affiliative behavior, milk let-down, and reproductive behavior (Gimpl and Fahrenholz, 2001). Oxytocin and the other neurohypophyseal peptides are evolutionarily conserved, and forms of this peptide family are found in all vertebrates and many invertebrates (Hoyle, 1999). For instance, the oxytocin-related peptide annectin is implicated in egg-laying behavior in earthworms (Oumi *et al.*, 1996). In Syrian hamsters, oxytocin promotes female receptivity by increasing the duration of lordosis in a dose-dependent manner (Whitman and Albers, 1995). In contrast, female prairie voles show decreased sexual receptivity, but enhanced social behavior associated with sexual receptivity, such as partner preference, with central injections of oxytocin (Witt *et al.*, 1990). In rats, central oxytocin administration initiates and maintains female sexual behavior (Arletti and Bertolini, 1985; Pedersen and Boccia, 2002). This trend across species for central oxytocin to increase female sexual behavior and decrease male sexual behavior is robust (Gimpl and Fahrenholz, 2001 and above), but there are notable exceptions (Kendrick *et al.*, 1993; Nishimori *et al.*, 1996; Gimpl and Fahrenholz, 2001; Argolias and Melis, 2004). For instance, oxytocin improves male

copulatory performance in rats and rabbits (Melin and Kihlstrom, 1963; Arletti *et al.*, 1985). Nevertheless, a strong case can be made for sexual dimorphism in many aspects of the oxytocinergic system in mammals.

Isotocin, the fish homologue of oxytocin (Murphy *et al.*, 1998), has received little study in terms of the regulation of behavior since the early reports of Pickford and Strecker (1977). They reported that peripherally injected isotocin had an effect on behavior, but it was simply due to minimal binding to arginine-vasotocin (AVT) receptors (Pickford and Strecker, 1977). Only recently has it been shown that the effect of central isotocin is distinct from the effect of vasotocin; there is a different effect of vasotocin versus isotocin on the neural activity that drives humming behavior in the plainfin midshipman fish (Goodson and Bass, 2000). Other recent work has shown that vasotocin and isotocin neuronal populations have distinct patterns of periodic  $\text{Ca}^{2+}$  pulses in rainbow trout, supporting separate signaling pathways for release of the two neuropeptides (Saito and Urano, 2001). In addition, isotocin receptors in the teleost *Catostomus commersoni* showed dramatically lower activation by vasopressin than by isotocin (Hausmann *et al.*, 1995). These three studies suggest that isotocin function is independent of the vasotocin neuropeptide pathway, both in its release and its downstream effects. This study examines these issues in *Lythrypnus dalli*, a teleost fish that is a useful model organism for looking at the links between socially-regulated behavior and neuropeptides.

The reproductive behavior of *Lythrypnus dalli* changes from female to male following the removal of a dominant male from a social group. This straightforward social manipulation predictably triggers the dominant female to change sex to male under both field (Black *et al.*, 2005; Chapter 2) and laboratory conditions (Reavis and Grober, 1999). The sex change process is characterized by a stereotyped behavior profile that allows changes in neuropeptides, such as

isotocin, to be mapped onto the sex change process (Fig. 3) (Reavis and Grober, 1999). Using this behavioral model, it is possible to examine the specific behavioral relevance of neurochemical changes over the course of sex change, in addition to providing basic comparisons among females, established males and ‘new males’ (*i.e.* females that have recently changed to males). I hypothesized that 1) there is a sexual dimorphism in isotocin peptide containing preoptic cells, and 2) social manipulation that leads to sex change would also change the isotocin system in the forebrain. I predicted that removal of male inhibition would change IST-ir neurons over the course of sex reversal in *Lythrypnus dalli* and, as a result, there would be a sex difference in IST-ir cells between males and females, with no difference between males and recently sex-changed fish.

## Methods

Experiments were conducted from July to August of 1997 at the Wrigley Institute for Environmental Studies at Catalina Island, California. *Lythrypnus dalli* were collected from the area around Bird Rock at depths of 6-12 meters (California Department of Fish and Game Permit #802002-02 methods as described in Chapter 2). After capture, all fish were placed in a large tank (59.1 cm W x 87.6 cm L x 30.5 cm D) housed outdoors, and later sorted by genital papilla shape into groups of one male and four females, with each fish identifiable by unique body markings (Reavis and Grober, 1999). The largest (focal) female was longer in standard length than any female in the group by 3 mm or more, and the male was 2 mm or more longer than the largest female. No individuals with a standard length of 20 mm or less were used. Individuals were given five days to adjust to their new social group in outdoor divided tanks (59.1 cm W x

17.5 cm L x 15.25 cm D or 29.5 cm W x 43.8 cm L x 15.25 cm D) provided with flow through seawater. Fish in all tanks were fed fish flakes twice daily. On the fifth day following group formation, males were removed from the social group to induce sex change in the largest female. Dominance of individual fish was determined by the frequency of jerk and displacement behavior and the individual's response to the jerks and displacements of other fish (Reavis and Grober, 1999; see Chapter 2). The behavior of the focal (dominant) female was recorded for fifteen minutes twice daily before the male was removed and then each subsequent day until focal female removal at stereotyped behavioral stages in the sex change process, described by Reavis and Grober (1999) and summarized below. Soon after male removal, focal females show an increase in displacement behavior and sometimes display jerk behavior. This is called the dominance phase. This is followed by dramatic decreases in jerk and displacement behavior that can last for a day or more, termed the quiescent period. The last stage is the courtship stage, when there is a second increase in jerk and displacement behavior before fertilizing eggs as a male. I sampled the brains and observed the genital papilla of focal females during the different behavioral stages of sex change: dominance, courtship, and after fertilizing eggs as a male (*i.e.*, sex-changed; Fig. 3). The first jerk behavior display was used to determine dominance phase, a 25% increase in displacement behavior following a previous 25% decrease from the initial peak rate of displacement behavior was used to determine courtship phase, and fertilization of eggs as a male was used to determine sex change. For sex changer groups, behavior was observed twice daily for 15 minutes until courtship phase, and then observers only looked for eggs fertilized by the dominant fish twice daily.

Focal females were removed following morning observations because of documented diurnal changes in plasma peptides of other fish species (Kulczykowska, 1998). After a dominant

female was removed from a group, the tank division was cleaned, a new group replaced the previous group in the division, and the entire process was repeated. Removed fish were euthanized with an overdose of MS-222 and immersed in fresh 4% paraformaldehyde for no less than a week with their eyes removed to allow adequate diffusion into the brain. Brains were dissected out from the skull and stored overnight at 4°C in 30% sucrose in 0.1M phosphate buffer for cryoprotection. Each brain was sliced in 20 µm sections on a cryostat, and every other section was labeled with oxytocin antisera (compliments of Park and Kawashima; Maejima *et al.*, 1994). During the immunocytochemistry, many brains from the initial sample were lost due to a batch of unsubbed slides. Because of this, there are higher sample sizes for the behavior data than the brain data and all traits discussed after the behavior data refer only to those fish that had brain data. In those brains, labeled cells in the forebrain preoptic area were quantified using NIH Scion Image1.62a (W.Rasband, NIH, Bethesda, MD). A set of control slides were double labeled for vasotocin and isotocin to assure the specificity of the oxytocin antisera for isotocin in *L. dalli*. All slides were coded so that the individuals who quantified the size and number of the cells did not know the identity of the fish to which each slide corresponded. The IST-ir cell size and number versus stage of sex change was compared using ANOVA. Because of the variation within groups, IST-ir cell size and number was also compared in the unequivocal males (control males and sex-changed new males) and unequivocal females (fish prior to courtship as a male) by use of a Mann-Whitney U Test. This comparison was done without correcting for body size and also correcting for body size by dividing the value by the standard length of the fish. All statistics were performed on StatView (v.5.0.1).

## Results

Prior to male removal, the largest female in the group had lower, but not statistically lower average daily displacements than the dominant male ( $0.776 \pm 0.116$  displacements/min for females versus  $1.106 \pm 0.207$  displacements/min for males, unpaired t-test,  $t=-1.505$ ,  $p=0.142$ ; Fig. 13). No females displayed jerk swim behavior prior to male removal, while males displayed an average of  $0.661 \pm 0.446$  jerks/min. Focal females showed an increase in displacement and jerk behavior followed by a decrease in both, and then another increase in the rates of both behaviors (Fig. 13) as seen previously in both the laboratory (Reavis and Grober, 1999) and field (Fig. 6). Rates of displacement were higher in this study than the field study (Chapter 2), but comparable to Reavis and Grober (1999). Courtship jerk swims were higher in this study than both the field study (Chapter 2) or Reavis and Grober (1999).

The average time for females to change sex upon removal of the male was  $7.8 \pm 1.3$  days ( $n=5$ ). For both IST-ir cell size and number, there was not a statistically significant difference across the different groups due to variation within groups and sample size (Kruskal-Wallis test,  $H=4.10$ ,  $p=0.39$  and  $H=6.36$ ,  $p=0.17$ , respectively) However, when comparing across the groups from control females through the stages of sex change to control males, the cell number was decreasing (Fig. 14). When unequivocal males and females were compared, females had more cells than males (Fig. 15A,  $p=0.013$ ). Correcting for body size, the difference was even larger ( $p=0.0092$ ). Thus, there appears to be a robust sexual dimorphism in IST-ir cell number in *L. dalli*. No significant difference was found in the cell size between males and females before (Fig. 15B,  $p=0.064$ ) or after correction for body size ( $p=0.297$ ).

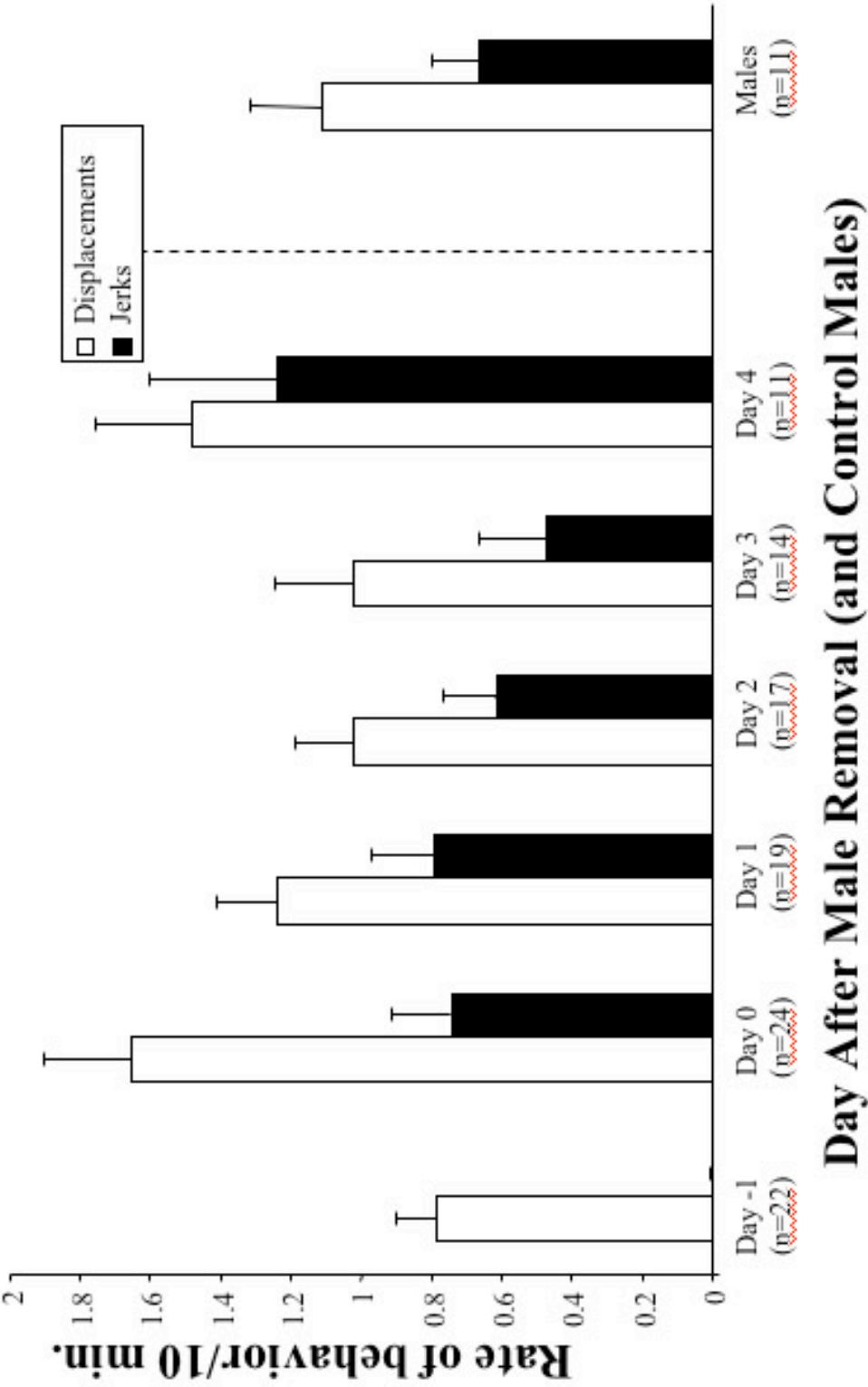


Figure 13: Jerk and displacement behavior of dominant males compared to the focal female prior to and in the first five days following male removal from the group in *L. dalli*. Fish with the highest initial (baseline) displacement behavior were selected as focal fish. Time from male removal to spawning as a male was 7-10 days, but behaviors were only recorded as an assay for different behavioral stages (after the start of courtship phase, behavior was not recorded). The sample size is decreasing as fish were being removed for different phases of sex change. Values are mean  $\pm$  SEM.



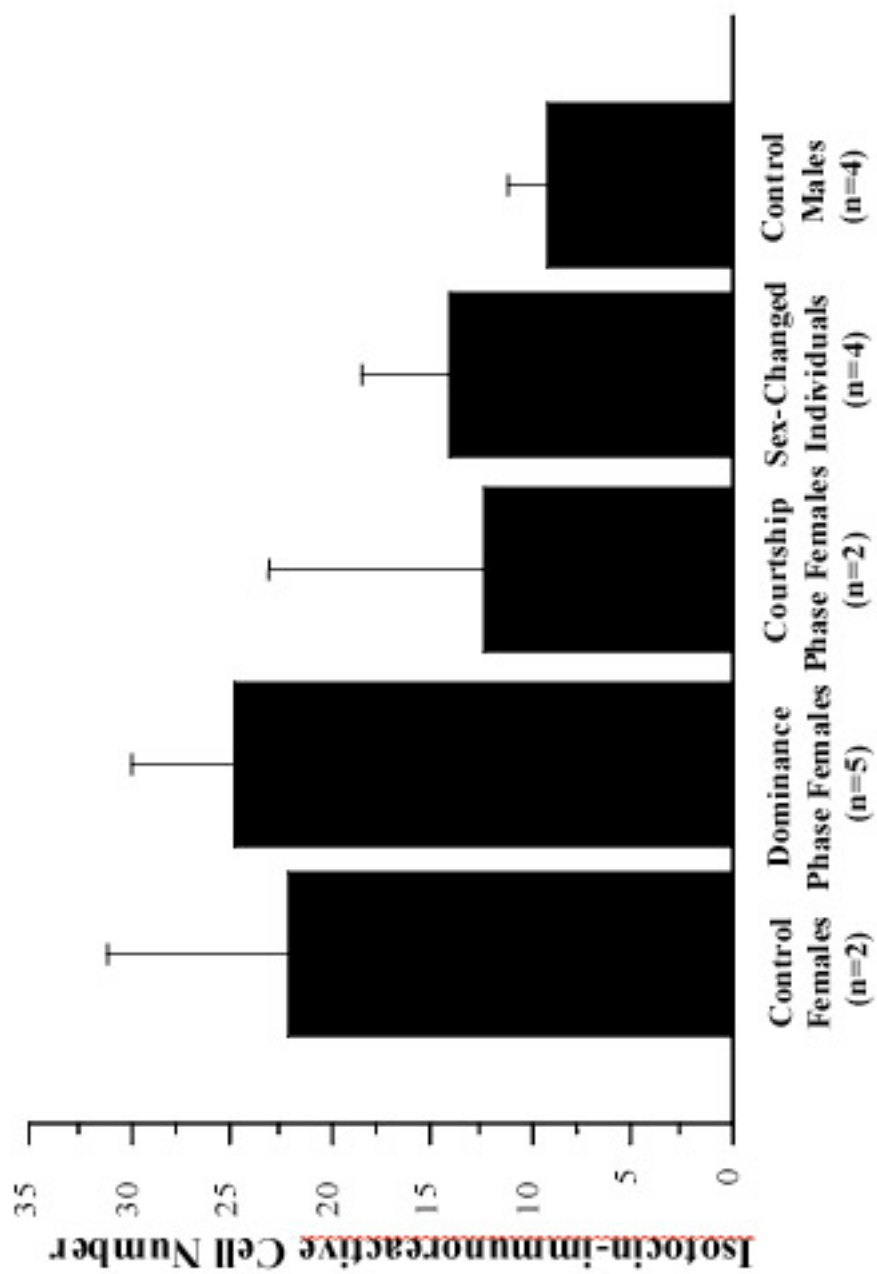


Figure 14: Sex change stage versus preoptic isotocin-immunoreactive cell number in *L. dalli*.

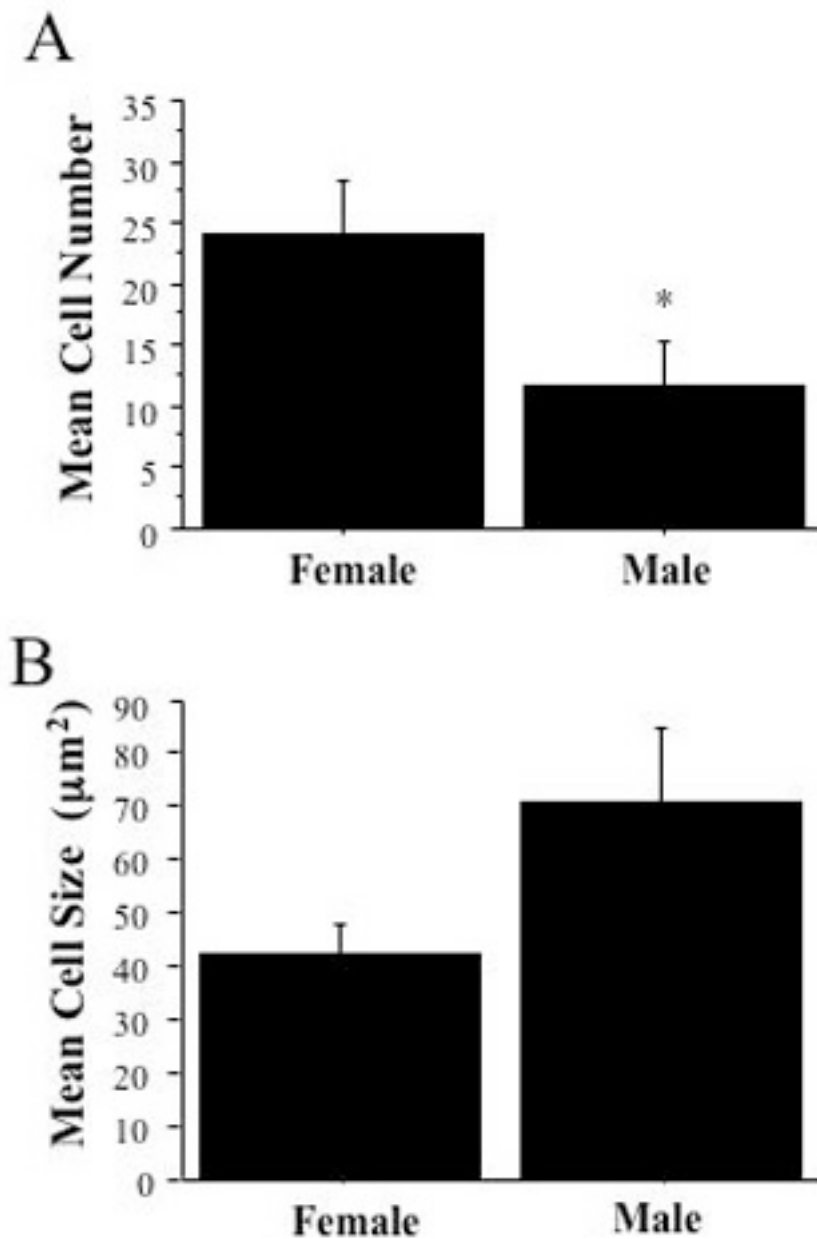


Figure 15: Differences in preoptic isotocin-immunoreactive cell number (A) (Mann Whitney U,  $p=0.013$ ) and size (B) (Mann Whitney U,  $p=0.067$ ) between *L. dalli* females (stages prior to courtship phase,  $n=7$ ) and males (control males and sex changed females  $n=8$ ). Error bars represent SEM. When body size is corrected for, the difference in cell number becomes greater ( $p=0.0092$ ) and the trend in size differences goes away ( $p=0.297$ ).

The cell number of each sex change stage in this experiment shows a great deal of variation (Fig. 14). The least amount of variation is seen in the number of IST-ir cells in the control males. Interestingly, the male with the highest number of cells (thus most female-like in cell number) had been seen with eggs the day before removal, but on the day of removal, this male did not have eggs in his nest tube.

Among females that had changed sex, the fish with the highest IST-ir cell number had a pointed but short genital papilla, while most of the others had transitional (longer) genital papillae. The sex changer with the lowest number of IST-ir cells had the only male-typical genital papilla of the group.

## Discussion

The removal of the male from a social group resulted in rapid sex change that was consistent in duration and pattern with previous studies in the lab (Reavis and Grober, 1999) and field (Chapter 2; Black *et al.*, 2005). This manipulation also resulted in a profound sex difference in IST-ir cell number. Females had more IST-ir cells than males, including males that had recently changed from female, yet there were no significant differences in cell size. The mammalian homologue for isotocin, oxytocin, (Murphy *et al.*, 1998) has been reported to inhibit male sexual behavior in prairie voles (Mahalati *et al.*, 1991). Oxytocin has also been shown to promote female sexual behavior in rats (Bale *et al.*, 2001) and other species as described above. The changes in isotocin in response to male removal may serve a similar reproductive function in *L. dalli*. The larger number of isotocin cells in females suggests that isotocin serves a modulatory function requiring more cells for repression of male function or enhancement of female function.

It is interesting to note that inhibition of isotocin with the selective oxytocin antagonist ((des-glycinamide<sup>9</sup>, d(CH<sub>2</sub>)<sub>5</sub><sup>1</sup>, O-Me-Tyr<sup>2</sup>, Thr<sup>4</sup>, Orn<sup>8</sup>)-vasotocin) resulted in a 300% increase in the number of fictive bursts by midshipman fish females (more male-like behavior) and infusion with isotocin decreased the number of fictive bursts and fictive burst durations in a dose-dependent manner (less male-typical behavior) (Goodson and Bass, 2000). These results suggest isotocin may be playing a similar behavioral role in midshipman fish.

An alternative hypothesis is that isotocin is playing a non-behavioral role, helping with normal female cycling and egg laying, that is not necessary when a fish changes to male. This would be consistent with oxytocin and related neuropeptides playing a physiological role in the production of offspring by females in both vertebrates and invertebrates (Moore *et al.*, 1992; Gimpl and Fahrenholz, 2001; Oumi *et al.*, 1996).

The male-like length to width ratio of the genital papilla is dependent on androgen levels (Carlisle *et al.* 2000), suggesting that the female with more IST-ir cells and a pointed but short genital papilla was not as far along as the others in the sex change process, even though it did fertilize eggs as a male. The sex changer with the lowest number of IST-ir cells had the only male-typical genital papilla of the group, further supporting this idea. Steroids have been shown to influence the oxytocin system in mammals (Jirikowski *et al.*, 1988; Johnson *et al.*, 1989), so this may be indicative of an androgen effect.

Because the “male” class also included males that had recently changed from female in this experiment, preoptic area isotocin-producing cells may die or be modified to no longer produce isotocin when a female changes to male. I have no current evidence on whether apoptosis has occurred or not. However, when males in the lab are artificially put together in a group with only larger males, they can revert back to females (Reavis and Grober, 1999). This

process takes significantly longer and the social situation is unlikely to occur in the field. The longer period of time to change from male back to female could be due to changes in the body and brain that are hard to reverse, such as cell death.

Control males may have the lowest amount of variation in IST-ir cell number due to these hard-to-reverse changes. The male with the highest IST-ir number lost its eggs before removal. Since males exclusively care for the eggs, perhaps this male ate its own eggs or did a poor job in keeping females from eating its eggs because its paternal behavior was less male-like.

The decrease in IST-ir cell number as a female changes to male is different from what is found in vasotocin-producing cells, where the cell number between males and females was found to be the same, but the cells grew larger as *L. dalli* changed from female to male (Reavis and Grober, 1999). The difference suggests that not only are isotocin and vasotocin serving different functions, but that they may be serving different sexually dimorphic functions. This idea is supported by work on another teleost fish, the plainfin midshipman, where vasotocin and isotocin had differential effects on fictive humming in males versus females (Goodson and Bass, 2000). Further studies using *in situ* hybridization to examine mRNA expression and behavioral observations coupled with injections of AVT and IST and their antagonists are needed in *Lythrypnus dalli* to examine how neuropeptide expression is changing in response to the social cue of male removal and what functions these neuropeptides may be serving.

This study provides what appears to be the first demonstration of a sexual dimorphism in the number of IST-ir cells in the preoptic area of a fish. Moreover, this is the first study to demonstrate changes in isotocin neurons in response to social modulation in any vertebrate. Removal of the male from a social group resulted in preoptic area IST-ir cells decreasing from female- to male-typical numbers in the dominant female as she changed sex. I suggest that *L.*

*dalli* provides a useful model system for investigating the role of neuropeptides in generating the sex differences that characterize most vertebrates, since these animals are sexually plastic during adult life and easily manipulated by changing the social environment.

## CHAPTER 4: SOCIALLY-REGULATED RAPID CHANGES IN AGGRESSION AND BRAIN AROMATASE ACTIVITY ARE CORRELATED

Figures and text modified from a version in review with Proceedings of the Royal Society of London, Series B: Black, M.P., Balthazart, J., Baillien, M., and Grober, M.S., Socially-mediated rapid changes in aggression and brain aromatase activity are correlated in a sex-changing fish, *Lythrypnus dalli*

Author roles: M.P. Black, J. Balthazart, and M.S. Grober designed the experiment and wrote the manuscript. M. Baillien ran the aromatase assays. All authors were involved in the data analysis.

### Summary

Social interactions can generate rapid and dramatic changes in behavior and neuroendocrine activity. I investigated the effects of a changing social environment on aggressive behavior and brain aromatase activity (bAA) in a sex-changing fish, *Lythrypnus dalli*. Aromatase is responsible for the conversion of androgen into estradiol. Male removal from a socially stable group of one male and three females resulted in rapid and dramatic ( $\geq 200\%$ ) increases in aggression in the dominant female, which will become male usually 7-10 days later. These dominant females and recently sex-changed individuals had lower bAA but similar gonadal aromatase activity (gAA) compared to control females, while bAA and gAA in established males were lower than those three groups. Within hours of male removal, dominant females' bAA but not gAA correlated with increases in aggressive behavior. These results are

novel because they are the first to: 1) demonstrate socially-induced differences in bAA levels that correspond with behavior, 2) identify this process as a possible neurochemical mechanism regulating the induction of behavioral, and subsequently gonadal, sex change, and 3) show differential regulation of bAA versus gAA resulting from social manipulations. Combined with other studies, this suggests that aromatase activity may modulate fast changes in vertebrate social behavior.

## Introduction

Aromatase, the enzyme that converts testosterone to estradiol, has been implicated in the early development and later adult expression of sexual behavior in a wide range of vertebrate species. Early treatments with aromatase inhibitors that generated rats with a bisexual phenotype (Bakker *et al.*, 1993) or reversed gonadal sex in chickens (Elbrecht and Smith, 1992) exemplify the important role of aromatase during sexual development. Aromatase in the brain also modulates behavior in adult birds and mammals (Lephart, 1996; Pinckard *et al.*, 2000). Most effects of estrogens derived from testosterone aromatization are thought to reflect specific changes in the transcription of estrogen-dependent genes (McEwen and Alves, 1999) but estrogen can have rapid actions through non-genomic mechanisms in a variety of biological systems (Kelly and Ronnekleiv, 2002) and its production can be rapidly regulated via changes in brain aromatase activity (AA) in birds (Balthazart *et al.*, 2001a,b, 2003a). Considering this fast regulation of brain AA and rapid estrogen effects on brain and behavior, I investigated a social trigger for rapid changes in sexual phenotype and its effect on AA and behavior. In a socially stable group of bluebanded gobies, *Lythrypnus dalli*, removal of the dominant male produces



dramatic behavioral and morphological modifications in the dominant female, which then changes sexual phenotype from female to male. Within minutes to hours of male removal, the dominant female increases her aggressive behavior, an accurate early indicator that the female will morphologically change sex to male (Reavis and Grober, 1999). This system therefore exhibits socially mediated transitions between sexual phenotypes and an early and unambiguous behavioral change that robustly predicts the individual that will change sexual phenotype.

In preliminary studies of *L. dalli*, males had lower AA in both brain and gonad than females, suggesting that a decrease in AA occurs during sex change. Changing social interactions in *L. dalli* could down-regulate AA (Grober, 1997) and cause a dramatic change in behavior (Reavis and Grober, 1999). To test this hypothesis, I compared AA and behavior of females in the early stage of sex change to control females, established males, and recently sex-changed fish.

## Methods

### *Subjects and in vivo manipulations*

Fish were collected off the coast of Catalina Island, California (permit #SC-003083; see Chapter 2), and then maintained in a fish facility in Atlanta, Georgia. The experiment ran from February-March of 2003 with 19 social groups. Each group had one large male ( $SL=37.27 \pm 0.53$  mm, means  $\pm$  SEM), one large female at least 3 mm smaller than the male ( $SL=30.85 \pm 0.61$  mm), and two females at least 3 mm smaller than the large female ( $SL=22.82 \pm 0.35$  mm). These sizes assured male dominance over all fish and the largest female's dominance over all females in the group. Each group was placed in a 40-liter aquarium with a PVC nesting tube and

given five days' adjustment to the new social conditions. On the fourth day, the largest female's behavior was observed for 10 minutes in both the morning and afternoon. Recorded behavior included approaches, displacements, and jerks (see Chapter 2; Reavis and Grober, 1999). Each behavior was averaged for the day as baseline frequency.

There were four group types: dominance phase, sex-changed, control females, and males. In dominance phase groups ( $n=8$ ), males were removed in the morning on the fifth day and a female's behavior was observed for 10 minutes after male removal, 10 minutes in the afternoon, and 10 minutes in the morning the next day. The large female in these groups was sacrificed after any of these three observation periods once she met behavioral criteria for dominance, as assessed by exclusive access to the nest tube and/or a doubling of her baseline frequency of aggressive displacement behavior. All dominance phase fish had been sacrificed after the third observation period. In each case, I recorded the time from when the male was removed from the social group to when the female's tissue was frozen (see below). These latencies are conservative time estimates because if a male had been in the nest tube, it may have taken some time before the female discovered that the male was missing. The dominance phase fish are at an early stage in the sex change process.

In sex-changed groups ( $n=4$ ), the male was also removed on the fifth day and the large female was allowed to fully change sex. Once it fertilized eggs as a male, it was sacrificed.

In control female groups ( $n=4$ ), the male remained in the group and the large female was sacrificed at the same time as in the sex changer groups. The sex changer and control groups were paired two by two before experiments began and the large females in these groups were sacrificed in parallel on the same days ( $8.5 \pm 2.53$  days) after male removal in the sex-changed groups.

To provide additional reference values, 6 males that had remained in control groups were also sampled at the same time as control females and their brains and gonads were collected for analysis. Four males came from the same groups as the 4 control females used in this study. The other two males came from control groups where “females” were excluded because gonad structure in females did not correspond with their genitalia (see below). No difference was detected between these two subgroups of males in brain or gonadal AA or last recorded displacements toward the dominant female (unpaired t-test,  $t=1.28$ ,  $p=0.27$  and  $t=1.40$ ,  $p=0.24$ ,  $t=0.27$ ,  $p=0.80$ , respectively).

At the conclusion of the study, a few subordinate females ( $n=5$ ) were taken from control tanks in a random fashion. The behavior of these fish was not observed, and they were taken out of curiosity rather than experimental design. Each subordinate was very close in size to the other small fish in the tank. The average difference in size from the other subordinate in the group was  $1.16 \pm 0.54$  mm.

All fish above were rapidly sacrificed via decapitation and the brain and then the gonad were removed and frozen on dry ice. Tissues were kept at  $-78^{\circ}\text{C}$  until shipping, on dry ice, to Belgium for AA assays.

The genital papilla (external genitalia) of each fish was photographed before and after the experiment and length:width ratios were measured (Carlisle *et al.*, 2000). The ratios were used to assess the subjects' sex at the experiment's start. However, the genital papilla of *L. dalli* is not a perfect predictor of functional sex (St. Mary, 1993). Before freezing, gonad inspection verified initial genital-based sex assignment and five fishes coming from 3 different groups were found to have gonads that were not consistent with the initial sex assignment. These groups were removed

from the experiment before I were aware of their brain or gonadal AA, their papillae data were not included, and numbers presented above correspond to the final sample sizes.

### *Aromatase assay*

All frozen brain and gonad samples were weighed, homogenized, and assayed for AA by measuring the tritiated water production from [ $1\beta$ - $^3\text{H}$ ]-androstenedione, as described by Roselli and Resko (1991), with minor modifications (Baillien and Balthazart, 1997). Homogenates containing about 1 mg of fresh weight tissue per assay were incubated with 25 nM androstenedione at 37°C for 1 hour for brain and 15 minutes for gonadal tissue. The incubation durations were selected based on preliminary experiments to limit the amount of substrate metabolized so that the enzymatic reactions could proceed linearly during the entire incubation period (data not shown). Preliminary assays had confirmed that the substrate concentration used here is saturating (at least 5 times  $K_m$ ) in *L. dalli* as it is in goldfish (Zhao *et al.*, 2001).

Within each experiment, controls using boiled brain or brain samples with an excess (final concentration 40  $\mu\text{M}$ ) of the potent and specific aromatase inhibitor, R76713 (Racemic vorozole, Janssen Pharmaceutica, Beerse, Belgium) never exceeded 300-600 dpm while active control samples had radioactivities ranging between 2,000 to 150,000 dpm. Assays were performed so that each run had controls and samples from each of the experimental groups. A recovery of  $93 \pm 2\%$  was usually obtained from samples of 10,000 dpm tritiated water conducted throughout the entire purification procedure (incubation, centrifugation, and Dowex column). Protein content of all homogenates was determined in triplicate by a micromodification of the Bradford method (Bradford, 1976). Enzyme activity was expressed in pmol/hr/mg protein

after correction of the counts for quenching, recovery, blank values, and percentage of tritium in  $\beta$ -position in the substrate.

### *Data analysis*

Statistics were performed using JMP 5.0.1, Statview 5.0, and SAS 8.02 (SAS Institute, Cary, NC). One-way ANOVA was used to compare differences between groups and was followed when appropriate by Fisher protected least significant difference tests to compare groups two by two. Simple linear regression was used to analyze relationships between AA levels, genitalia, behavior, and latency between male removal and sacrifice. All data in the text are presented as means  $\pm$  SEM.

## **Results**

### *Morphologic Traits*

The gonadosomatic index (GSI), or the proportion of gonad mass to body mass (gonad mass/body mass times 100), was different between the groups ( $F_{4,23}=6.249$ ,  $p<0.01$ ; Fig. 16). Control females were different from control males ( $p<0.05$ ), but no other groups in GSI ( $p>0.05$ ). Dominance phase females, sex changers and males were not significantly different from each other in GSI ( $p>0.05$ ), but all differed from subordinate female fish GSI ( $p<0.05$ ).

Initially the genitalia of all females were not significantly different from each other ( $p>0.05$ ) and all were different from the genitalia of the males in terms of genital papilla ratio ( $F_{4,23}=17.34$ ,  $p<0.05$ ; Fig. 17). At the experiment's end, sex-changed individuals had genital

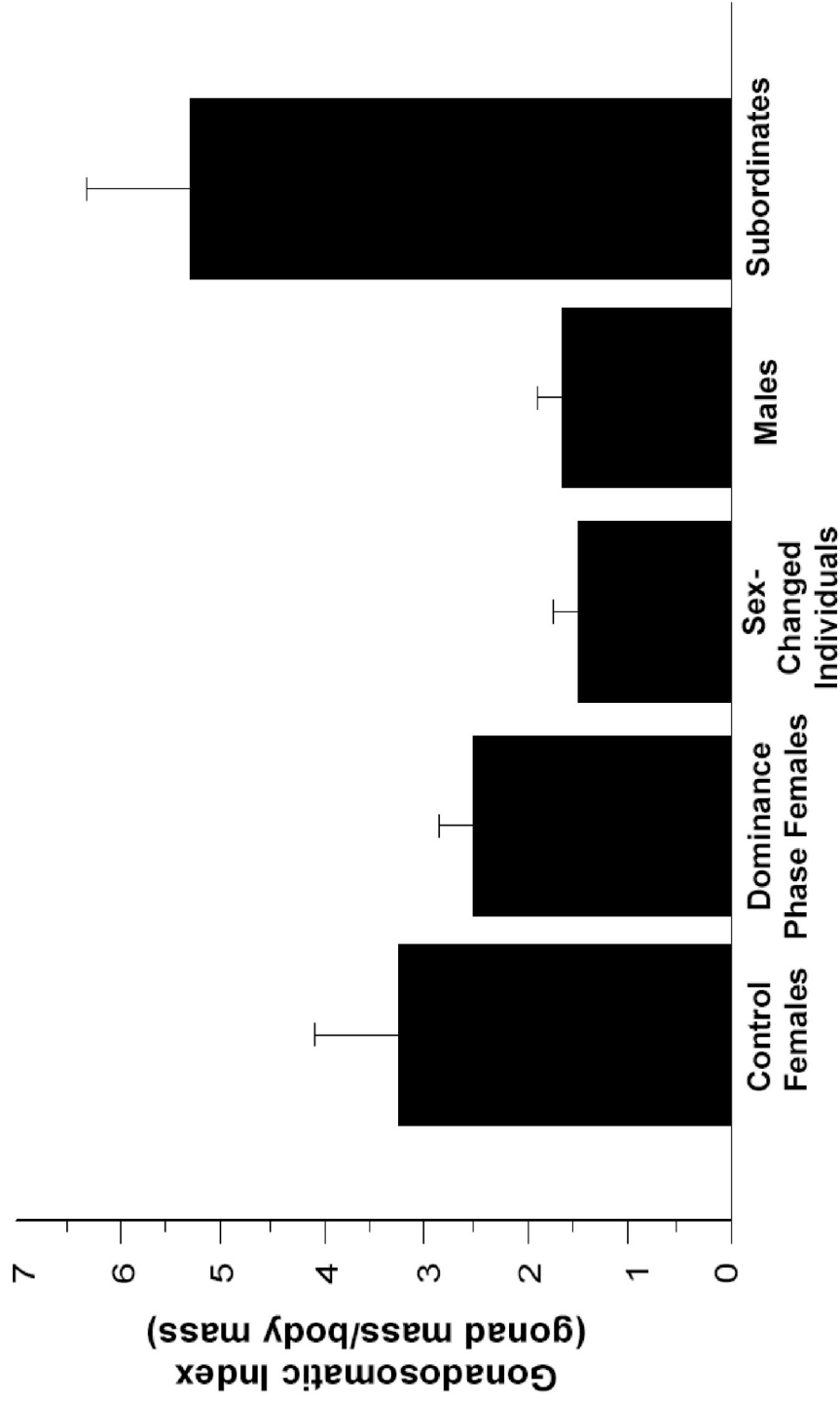


Figure 16: The gonadosomatic index (gonad mass/body mass x 100) for *L. dalli* was different between the groups ( $F_{4,23}=6.249$ ,  $p<0.01$ ). Control females ( $n=4$ ) were different from control males ( $n=6$ ;  $p<0.05$ ) but not different from other groups in GSI ( $p>0.05$ ). Dominance phase females ( $n=8$ ), sex changers ( $n=4$ ), and males were not significantly different from each other in GSI ( $p>0.05$ ), but all differed from subordinate fish ( $n=6$ ) GSI ( $p<0.05$ ).

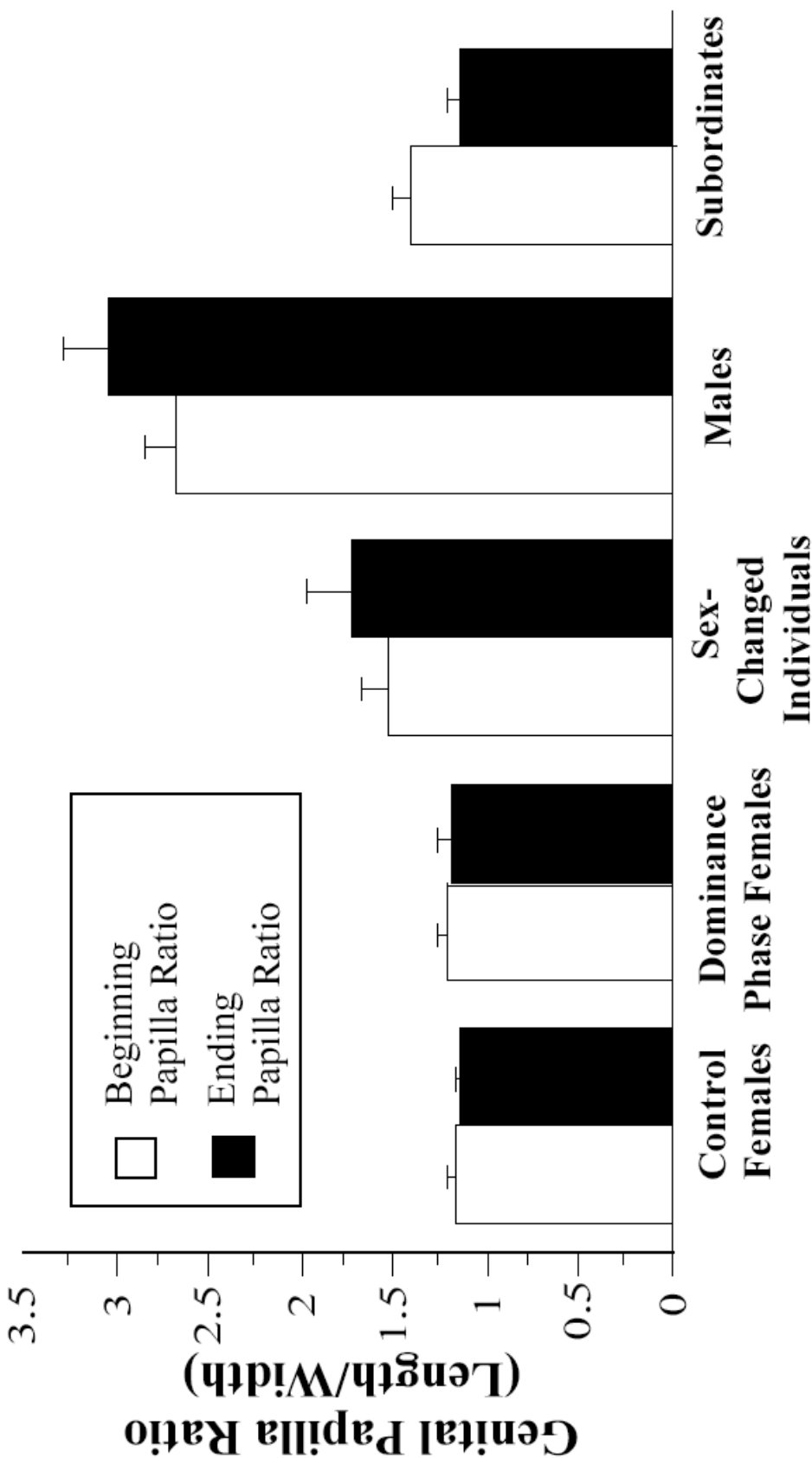


Figure 17: *L. dalli* genital papilla (genitalia) length:width ratio at the beginning of the experiment (white) and at sacrifice (black). Initially the genital papilla ratios (GPR) of all females were not significantly different from each other ( $p > 0.05$ ) and all were different from the GPR of the males ( $F_{4,23} = 17.34$ ,  $p < 0.05$ ). At the experiment's end, sex-changed individuals had GPR values in between those of males and females and significantly different from both males and females ( $F_{4,20} = 32.34$ ,  $p < 0.05$ ). Males and females were significantly different from each other ( $p < 0.05$ ).

papilla ratios in between those of males and females and significantly different from both males and females ( $F_{4,20}=32.34$ ,  $p<0.05$ ). Males and females were significantly different from each other ( $p<0.05$ ).

### *Behavior*

Baseline frequency of aggressive behavior in the largest female of the group was not different between the three female groups before male removal (Kruskal-Wallis test,  $DF=2$ ,  $p=0.65$ ; Fig. 18A). As expected from Reavis and Grober (1999), sex changers and dominance females were not statistically different from each other, but they were both different from control females when comparing changes in aggression (aggression at sacrifice minus aggression with the male present; ANOVA,  $F_{2,15}=4.21$ ,  $p<0.05$ , followed by Students'  $t$ ,  $p<0.05$ ; Fig 18A).

### *Brain aromatase*

Dominance phase females had their brains frozen on dry ice an average of  $12.76 \pm 4.65$  hr after male removal. This relatively large average latency is due to the fact that some subjects went through two observation periods without meeting criteria, and so had to be kept overnight for a third observation period the following day. Thus, although most groups reached criterion in the first 10-min observation period (62.5%), the median time to collect brain tissue was about 3.79 hr after male removal. All groups that reached criterion in the first observation period were frozen less than 4.25 hr after male removal.



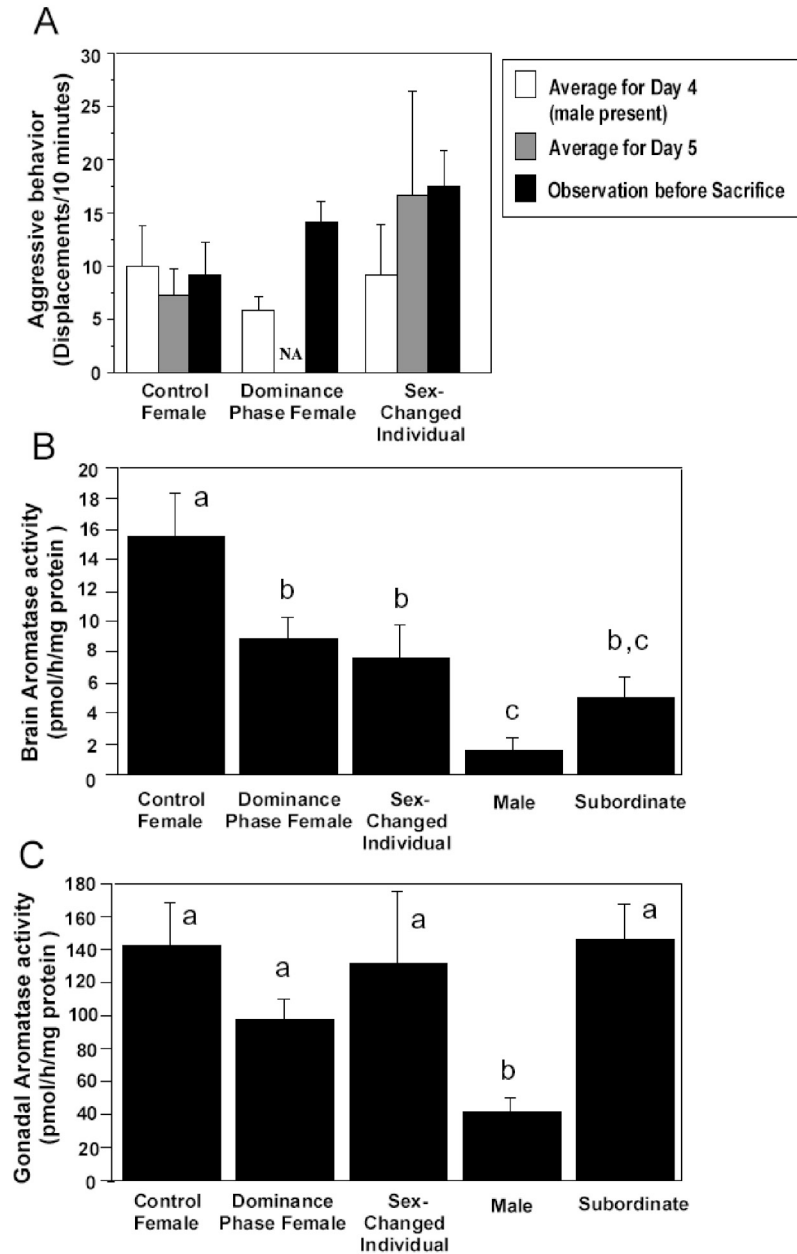


Figure 18: Aggressive displacement behavior and aromatase activity (AA) in the brain and gonad of *L. dalli*. For behavior (A), on Day 4 (prior to male removal), there was no statistical difference in average daily displacements (baseline frequency) between the largest females. On Day 5, the male was removed from dominance phase, and sex-changed groups and dominant females increased their aggressive behavior. Dominance phase fish have no average (NA) Day 5 data because they were sacrificed during Day 5 or just after. Brain (B) but not gonadal (C) AA was significantly lower in dominance phase and sex-changed individuals compared to control females, yet both brain and gonadal AA was higher in all female types than males (second and third panels). Different letters above the bars denote statistical differences ( $p < 0.05$ ) based on post hoc Fisher PLSD tests following a significant overall ANOVA.

Brain AA (bAA) differed markedly among the experimental groups ( $F_{4,23}=9.16$ ,  $p<0.001$ ; Fig. 18B). BAA was significantly higher in control females than in the early dominance phase females ( $p=0.01$ ) and sex changers ( $p<0.01$ ). The early dominance females were not different from the sex changers. In addition, males had lower bAA than all groups ( $p<0.05$ ) except subordinate females, which were not different from any of the experimental groups ( $p>0.05$ ).

### *Gonadal aromatase*

Gonadal AA (gAA) was significantly different between groups ( $F_{4,23}=4.51$ ,  $p<0.01$ ) with significantly lower gAA in males than in all other groups. There was no significant difference between the other groups, including those that were in the process of changing sex or had just changed sex and fertilized eggs as a male (Fig. 18C). Because testicular tissue is built up faster than ovarian tissue is broken down, ovarian tissue remains in fish that fertilized eggs and behaved as a male (Chapter 2; Black *et al.*, 2005). Visual inspection of gonads confirmed that sex-changed individuals still showed both ovarian tissue and testicular tissue, while males did not have any visible ovarian tissue.

### *Correlations*

One intriguing result was that bAA was not correlated with the amount of time after male removal ( $R^2<0.001$ ,  $p=0.99$ ), but rather with the increased aggressive behavior of dominance phase fish (Fig. 19;  $R^2=0.62$ ,  $p=0.04$ ,  $n=7$  because one of the 8 females dominated the nest but didn't show an increase in aggressive behavior and had an aromatase activity level, within one

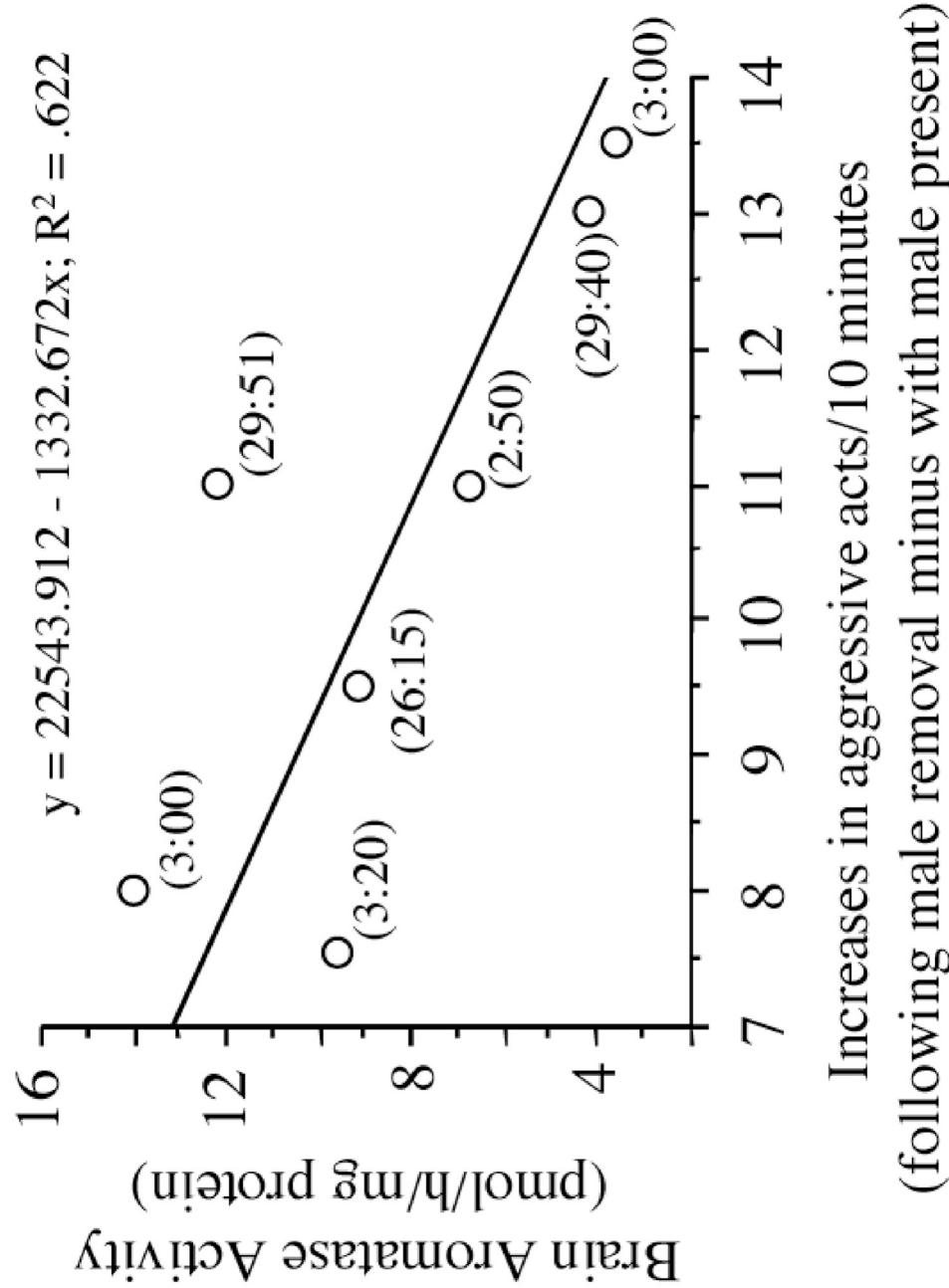


Figure 19: Correlation between aromatase activity and socially-induced increases in aggression in *L. dalli* during the dominance phase of sex change. Increased aggression is scored as the number of aggressive acts (displacements) performed after male removal during the last 10-min test period before sacrifice minus the number of acts performed in the presence of the male (prior to male removal). The number next to each data point represents the time from male removal (the social cue) to when the brain was frozen on dry ice (e.g. 3:20 is 3 hr and 20 min).

standard deviation of the control female average, 11.473 pmol/hr/mg protein). Moreover, the level of aggression of observed fish both prior to removal of the male and just before sacrifice varied from fish to fish but neither correlated with bAA ( $R^2=0.02$ ,  $p=0.71$  and  $R^2=0.21$ ,  $p=0.25$ , respectively). It was the increase in behavior following male removal that correlated with lower bAA.

The final genitalia length:width ratio of all fish sacrificed did not correlate with bAA or gAA ( $R^2<0.03$ ). GAA was not correlated with bAA or the increase in aggression of dominance phase fish ( $R^2<0.03$ ,  $p>0.41$ ).

## Discussion

This study found higher bAA in females than males in *L. dalli*, contrary to what has been observed in birds and mammals (Schumacher and Balthazart, 1986; Roselli, 1991; Lephart, 1996), but consistent with some fishes (*e.g.* Callard *et al.*, 1978; Contractor *et al.*, 2004; but see Gonzales and Piferrer, 2003). More importantly, removal of the male results in a rapid increase in aggression in the largest female, correlating with lower bAA but not gAA within hours. The female that establishes dominance through this increased aggression will fertilize eggs as a male often within 7-10 days, but as a newly sex-changed individual it has similar bAA and gAA levels to dominance phase individuals. However, in established males, bAA and gAA are significantly lower than in sex-changed individuals that have recently changed sex from female to male. These results are novel in that they are the first to: 1) demonstrate socially-induced differences in bAA levels that correspond with behavior, 2) identify this process as a possible neurochemical

mechanism regulating the induction of behavioral, and subsequently gonadal, sex change, and 3) show differential regulation of bAA versus gAA resulting from social manipulations.

### *Differences between brain and gonad*

The finding that bAA and gAA are differentially regulated is not unexpected. Several mechanisms are available to differentially regulate AA in the brain and gonad. In goldfish and zebrafish, the aromatase *CYP19B* gene is expressed more in the brain, while *CYP19A* predominates in the gonad, so differing expression of these two genes could explain the difference between bAA and gAA (Callard and Tchoudakova 1997; Tchoudakova and Callard, 1998). Moreover, tissue-specific promoters can differentially regulate aromatase expression in mice and humans (Simpson *et al.*, 2000). On a shorter-term basis, it has been shown in quail that brain and ovarian aromatase react differentially to calcium and various phosphorylating conditions (Baillien M. and Balthazart J., unpublished data), so there could be rapid local changes regulating AA.

### *Is a decrease in brain AA the cause of the behavioral sex change?*

Whatever mechanism mediates the changes (Balthazart and Ball 1998), the data suggests that early in the process of brain reorganization from female to male (to the dominant individual of the group), bAA drops dramatically. A decreased bAA in *L. dalli* should limit estrogen synthesis, leave more testosterone available for conversion into 11-ketotestosterone (11-KT), a potent fish

androgen (Borg, 1994; Fig. 20), and thus increase the brain androgen:estrogen (A:E) ratio. Such an increased A:E ratio (or an increase in 11-KT production alone) may be responsible for the increased aggression that negatively correlates with bAA. In fish, behavior can change rapidly in response to 11-KT or 17 $\beta$ -estradiol (Remage-Healey and Bass, 2004), and there is some evidence that increased production of androgen, such as 11-KT, is associated with aggression (e.g., Brantley *et al.*, 1993; Borg, 1994). Moreover, similar negative correlations between bAA and aggression have been observed in mammals. For example, in *Peromyscus* mice, increases in aggressive behavior correlate with reduced bAA in the bed nucleus of the stria terminalis, and experimentally reduced aromatase levels resulted in shorter attack latencies (Trainor *et al.*, 2004).

#### *Differences between sex-changed fish (new males) and established males*

A rapid drop in brain AA in the largest female could quickly increase her aggression levels to maintain or establish dominance over other females, thereby keeping them from also initiating sex change. Androgens produced by new testicular tissue could then provide positive feedback toward changing sex, while ovarian tissue is degraded later.

The ovarian tissue remaining in sex-changed fish may also regulate bAA through estrogen production. Since estrogen up-regulates bAA in other fishes (Pasmanik *et al.*, 1988; Kishida and Callard, 2001), ovarian estrogen may prevent a drop to bAA levels observed in males that have degraded their ovarian tissue completely. The subordinate fish pose a challenge to this idea because they had the highest gAA, yet their bAA was not significantly different from males. It was, however, high enough not to be different from dominance phase females or recently

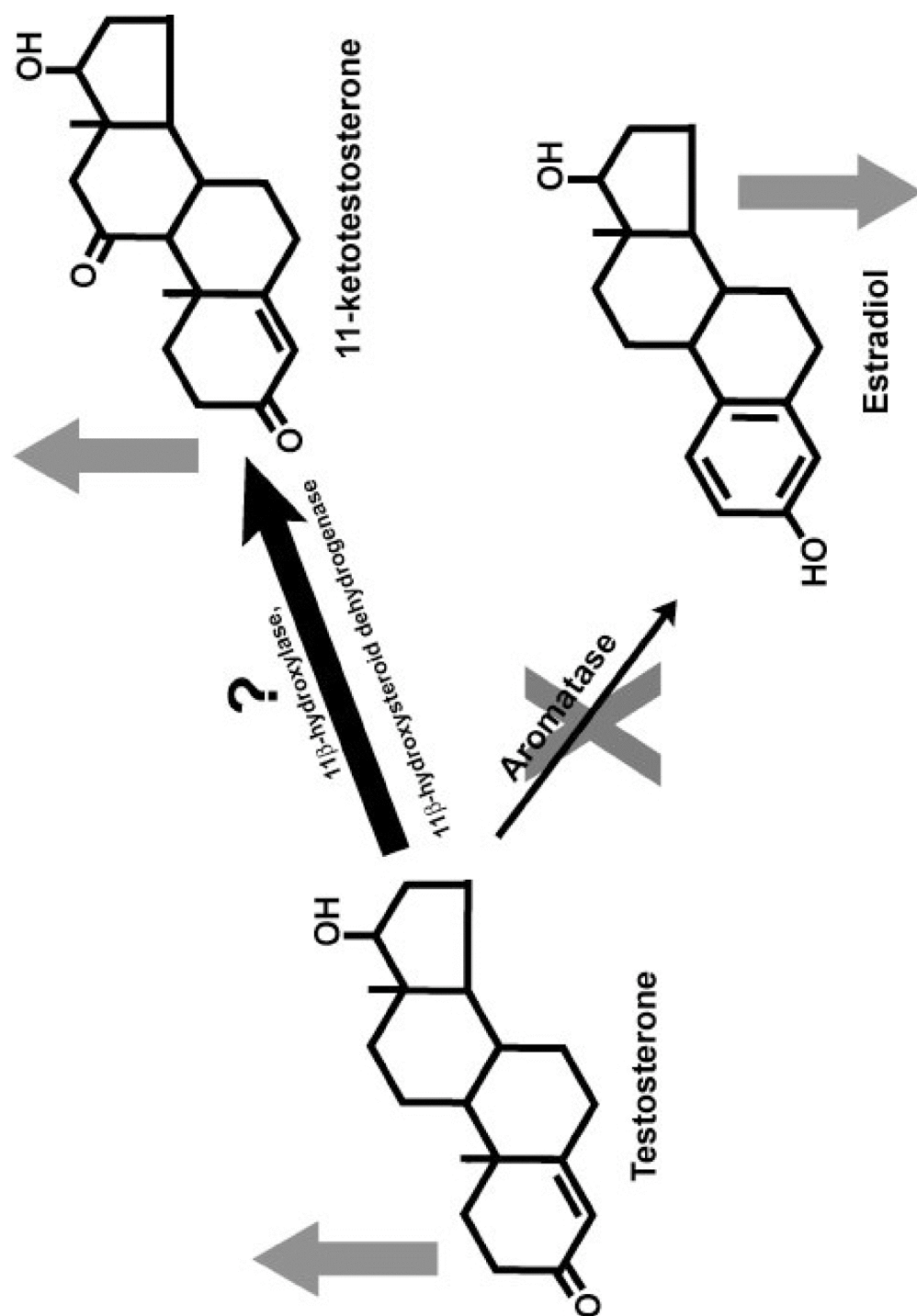


Figure 20: Model for the potential neurosteroidal consequences of decreased aromatase activity in *L.dalli* (note gray X). First, estrogen production decreases, while testosterone levels increase (indicated by gray arrows). Higher levels of testosterone (T) substrate could (see question mark) then increase conversion to 11-ketotestosterone (11-KT; gray arrow). The increased T and/or greater conversion to 11-KT, reduced estrogen, or the greater androgen:estrogen ratio could be affecting the brain, behavior and morphology of sex changing individuals.

sex-changed fish, so perhaps estrogen from the gonad is affecting brain aromatase activity. The lower aromatase activity may have been due to more social challenges in the subordinates than the dominant fish. Because I had not designed the experiment to look at the subordinates and the remaining fish available to me at the time were all small, the subordinates were unintentionally close in size. When subordinates are close in size, they tend to challenge each other more often and jockey for the social position more than if there were large size differences (Black and Grober, personal observation). This added competition between subordinates may have decreased their bAA and increased their aggressive behavior towards each other. Since I did not focally observe subordinate fish, I cannot address this question.

*Is a decrease in brain AA the cause of the morphological sex change?*

As changes in bAA can affect peripheral levels of steroids in the zebra finch (male estrogen levels; Schlinger and Arnold, 1991), the lower bAA in dominance phase and sex-changed fish may have been sufficient to affect peripheral levels of androgens. Androgens are known to promote traits observed in this study, such as testicular growth, secondary sex characters like the accessory gonadal structure, and increases in genitalia length:width ratios in *L. dalli* (Carlisle, 2001). Morning samples of free 11-KT positively correlated with the percent of male tissue in the gonad, with the size of the accessory gonadal structure and with aggressive displacement behavior on the last day before the fish were sacrificed in sex changers in the field (Chapter 2; Black *et al.*, 2005).

The known effects of androgens on morphology combined with the results of this study that suggest bAA drops prior to gAA during sex change lead to the intriguing possibility that a



change in the social environment causes early down-regulation of AA in the brain, which triggers a cascade of events resulting in morphological sex change. In short, the brain leads the gonad in this process (*e.g.* Grober and Bass, 1991; Francis, 1992). This mechanism is consistent with studies showing that bluehead wrasse behaviorally change sex in the absence of their gonads and gonadally derived steroids (Godwin *et al.*, 1996). This is also consistent with a proposed model of down-regulation of AA driving behavioral changes that independently precede gonadal changes (Grober, 1997). Further, if the brain is a source of androgen either by *de novo* production or rapid changes in steroid synthesis, the brain rather than the gonads could be the source of rapid changes in plasma steroids following challenge. As noted by Remage-Healey and Bass (2005), there have been many experiments documenting rapid changes in plasma steroid levels in response to social challenge or sexual stimuli in rodents (Batty, 1978; Macrides *et al.*, 1975), humans (Bernhardt *et al.*, 1998; Roney *et al.*, 2003), lizards (Greenberg and Crews, 1990; Yang and Wilczynski, 2002), birds (Wingfield, 1985; Wingfield and Wada, 1989), and fish (Cardwell and Liley, 1991a; Oliveira *et al.*, 1996). However, the definitive source of the rapidly elevated steroids has not been identified in these studies. In the male song sparrow, *Zonotrichia melodia*, Wingfield and Wada (1989) even showed that minutes after an intruder, a rise in testosterone preceded a rise in lutenizing hormone, supporting the idea that the peripheral testosterone might come from an extragonadal source such as the brain.

While it is possible that peripheral levels of steroids in *L. dalli* might be affected by bAA or that lower bAA could trigger a cascade of events resulting in morphological sex, the low levels of bAA in subordinate females of control groups provides evidence that low bAA does not automatically and irreversibly initiate sex change. There are many potential reasons subordinate fish might have low bAA levels and not change sex. It may be that subordinate fish have lower

initial testosterone production than dominants. There are likely to be higher levels of cortisol production in subordinate fish due to their social status, as seen in many subordinate vertebrates that do not cooperatively breed (reviewed in Creel, 2001). Conversion of 11-deoxycortisol to cortisol or metabolizing cortisol into an inactive form, cortisone, uses 11 $\beta$ -hydroxylase and 11 $\beta$ -hydroxysteroid dehydrogenase, respectively, the same two enzymes that are used to convert testosterone into 11-ketotestosterone (Perry and Grober, 2003; Fig. 21). It may be that decreased aromatase activity in the subordinates results in higher testosterone availability, but the enzymes are flooded by 11-deoxycortisol and/or its product cortisol and only very little testosterone is converted into 11-KT. Even without competition for the enzyme, there may simply be lower levels of 11 $\beta$ -hydroxylase and/or 11 $\beta$ -hydroxysteroid dehydrogenase enzyme activity available in subordinates than there are in dominants to make 11-KT from testosterone. Without the dramatic conversion to 11-ketotestosterone, a low level of aromatase may provide more testosterone but have no effect on whether these fish change sex. More studies are needed to determine what is happening in the subordinate females, looking at the behavior, brain, and plasma steroid levels, as well as levels of activity of different steroid metabolizing enzymes such as 11 $\beta$ -hydroxylase and 11 $\beta$ -hydroxysteroid dehydrogenase.

Steroids have potent effects in sex changing fishes and aromatase activity in the body may have a profound effect on the sex change process. Sexual phenotype differences in AA are found in several fishes (*e.g.*, Schlinger *et al.*, 1999), including sex changers (*e.g.*, Kincl *et al.*, 1987, Lee *et al.*, 2002). Blackeye gobies, *Rhinogobiops nicholsii*, changed sex from female to male when treated with 11-KT or with aromatase inhibitor (Kroon and Liley, 2000). Similarly, aromatase inhibitors blocked protandrous (male to female) sex reversal in the black porgy (Lee *et*

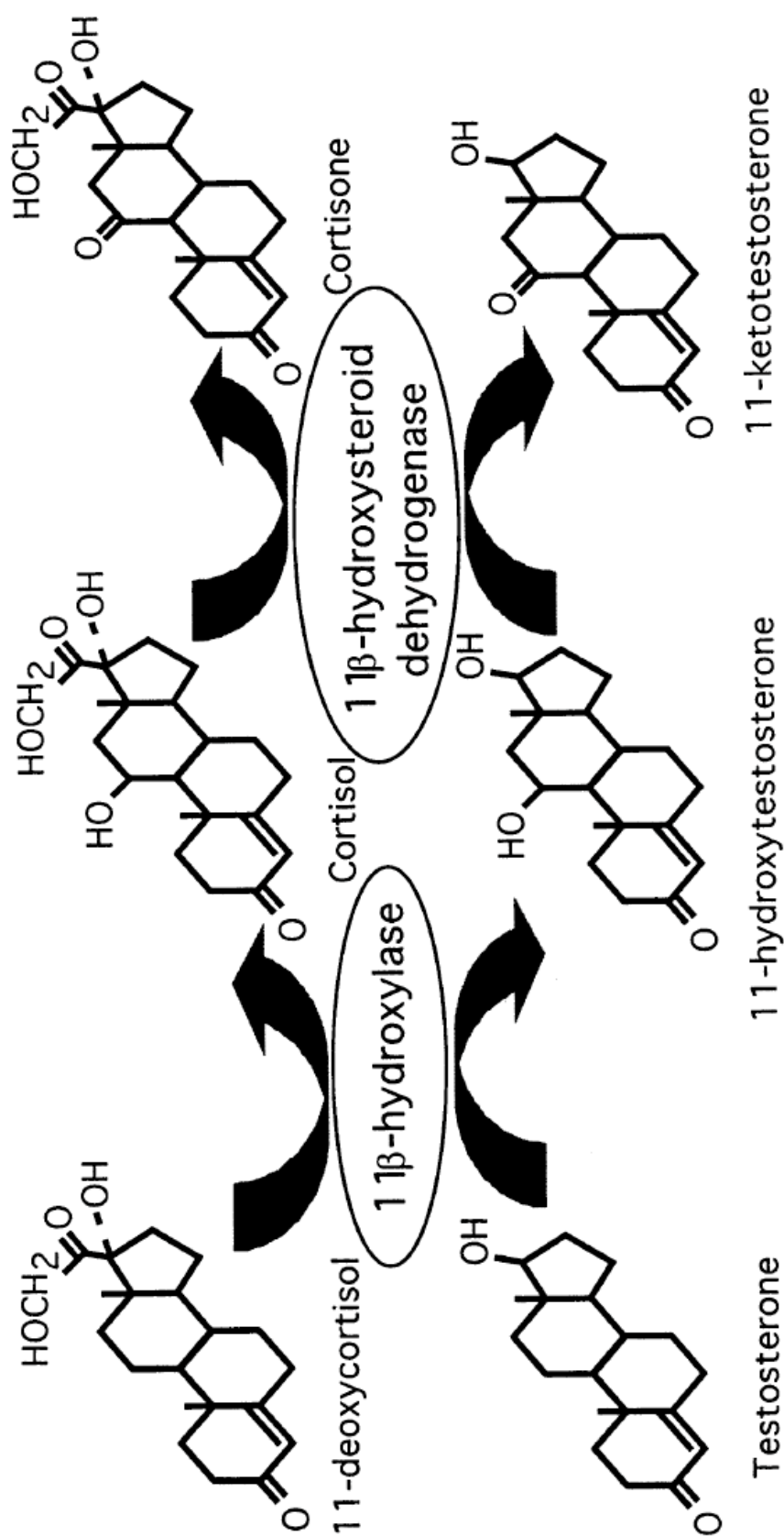


Figure 21: Pathways of glucocorticoid and androgen synthesis and inactivation in fish. The enzymes 11 $\beta$ -hydroxylase and 11 $\beta$ -hydroxysteroid dehydrogenase are responsible for glucocorticoid synthesis and inactivation, as well as 11-ketotestosterone synthesis. The common use of these enzymes creates the potential for competitive inhibition by the substrates and products of these two steroidogenic pathways. (Figure and legend reprinted with permission from Perry and Grober, 2003 and Elsevier).

*al.*, 2002). These studies implicate aromatase's role in the sex change process, but do not identify the exact nature or timing of that role.

Future studies in *L. dalli* will focus on what mechanisms decrease bAA, what the observed bAA levels in subordinates means, the effect of various steroid profiles on brain, behavior and sex change, how quickly bAA changes in the largest female following male removal, and whether a causal relationship between bAA, aggression and sex change exists.

## **CHAPTER 5: RAPID BEHAVIORAL CHANGES PRECEDE CHANGES IN BRAIN AROMATASE ACTIVITY DURING SOCIALLY-MEDIATED SEX CHANGE**

Author roles: M.P. Black, J. Balthazart, M. Baillien, and M.S. Grober designed the experiment. M. Baillien and M.P. Black ran the aromatase assays. M.P. Black and M.S. Grober were involved in the data analysis and wrote this chapter.

### **Summary**

The ability to respond appropriately and quickly to a social cue is profoundly important for an individual of a highly social species. Previous work suggested that a rapid response made by the social fish, the bluebanded goby, *Lythrypnus dalli*, could be coordinated by rapid changes in aromatase activity in the brain (bAA). To test the idea that rapid changes in bAA led to rapid increases in aggression, this experiment used a more rapid time course of behavioral profiling and bAA assay. The bAA of control females with a male in their group was compared with the bAA of fish that doubled their aggressive behavior by 10, 20 or 30 minutes after the male was removed from the social group and those that did not double their aggressive behavior by 30 minutes. The results demonstrate that changes in bAA do not precede increases in aggressive behavior and that bAA and aggressive behavior are not correlated on a time scale of minutes. In conclusion, whole brain aromatase activity decreases are not the cause of the rapid increases in aggressive behavior following male removal.

## Introduction

Social interactions often require fast responses. By acting first, an individual can influence the neurochemical and physiological state of others in a social group and therefore influence the behavioral state of others in the group (Summers, 2001). However, the mechanisms by which an organism can rapidly respond to social cues are currently not well understood. One potential way is by rapidly modulating steroid levels (Wingfield *et al.*, 1990).

Aromatase activity can rapidly (within minutes) decrease or increase in birds (Balthazart *et al.*, 2001a,b, 2003a). These rapid changes have also been correlated with changes in behavior linked to the production of estrogen via conversion from testosterone in quail (Evrard and Balthazart, 2004a, 2004b). Further, the Gulf toadfish can rapidly (within 5-20 minutes) change 11-ketotestosterone (11-KT) levels and vocalization behavior in response to simulated territorial intrusion, and in midshipman fish, exogenous application of 11-KT or 17 $\beta$ -estradiol results in rapid changes in calling behavior (Remage-Healy and Bass, 2004, 2005). These studies demonstrate that steroids can change very rapidly, that changes in steroid levels can cause fast changes in behavior, and that social changes can modify both the changes in steroid levels and behavior.

In a previous experiment (Chapter 4), I found that a few hours following removal of the male, large dominant females had lower levels of brain aromatase activity (bAA) than large females with the male still present in their social group. Further, I found that the changes in aggressive displacement behavior of dominant females negatively correlated with their bAA levels. One hypothesis that emerges from these results is that decreasing brain aromatase activity

levels are causing a change in the aggressive behavior of dominant fish. This experiment was designed to test that hypothesis.

## Methods

Fish were captured off the coast of Catalina Island, California (permit #SC- 801200-01; see Chapter 2), and then maintained in a fish facility in Atlanta, Georgia. Groups were tested from July 2003 to January 2004. Each group had one large male ( $SL=35.46 \pm 0.33$  mm, means  $\pm$  SEM), one large female at least 3 mm smaller than the male ( $SL=29.76 \pm 0.31$  mm), and two females at least 3 mm smaller than the large female ( $SL=24.15 \pm 0.22$  mm). These sizes assured male dominance over all fish and the largest female's dominance over all females in the group. Each group was placed in a 40-liter aquarium with a PVC nesting tube and given five days' adjustment to new social conditions. On the fourth day, the largest female's behavior was observed for 10 min in both the morning and afternoon. Recorded behavior included approaches, displacements, and jerks (see Chapter 2; Reavis and Grober, 1999). Each behavior was averaged for the day as baseline frequency. In experimental groups, the male was removed around 9:00 on the fifth day, and the dominant female was observed in consecutive 10-min time periods until it showed a doubling of displacement behavior relative to pre-removal baseline frequency or until 30 min had passed without the doubling in behavior. The dominant female was sacrificed at a point when one of those criteria was met. Fish generally doubled their baseline behavior within the first 10 min ( $n=13$ ) or did not double their baseline behavior by 30 min (30 NC group,  $n=8$ ), so the sample sizes for 20- and 30-min fish are low ( $n=6$  and  $n=4$ , respectively). Three fish did not appear to display dominance and were excluded from all analyses. Another group of sex

changers was not collected until they fertilized eggs as a male (n=13), and a group of males (n=11) was collected for comparison as well.

The genital papilla (external genitalia) of each fish was photographed before and after the experiment and length:width ratios were measured (Carlisle *et al.*, 2000). The ratios were used to assess the subjects' sex at the experiment's start. However, the genital papilla of *L. dalli* is not a perfect predictor of functional sex (St. Mary, 1993). Before freezing, gonad inspection verified initial genital-based sex assignment, and two fish coming from two different groups were found to have gonads that were not consistent with the initial sex assignment. These fish were removed from the experiment before I was aware of their bAA. Gonadosomatic index (GSI) was calculated by taking the ratio of gonad mass to the mass of the fish and multiplying it by 100,

Most of the sex changers, males, and one female were in a box together that was held by Fed Ex for longer than expected. The dry ice sublimated completely, and although the box was still cold on arrival, the samples had melted during shipping (as evidenced by the tissue being stuck to the tubes). For this reason, the brain aromatase activity levels of these fish may not be reliable. As these were not essential to evaluating differences in the largest females immediately following male removal, the males and sex changers are included for comparison, but no melted samples were included in the statistics or discussed in relation to the others' AA results. The bAA datum for the one female from that box was not included. All frozen brain and gonad samples were homogenized and assayed for AA as described in Chapter 4.

Analyses were performed using Statview 5.0.1 and JMP 5.0.1 (SAS Institute, Cary, NC). One-way ANOVA was used to compare differences between groups and was followed when appropriate by Fisher protected least significant difference tests to compare groups two by two. Simple linear regression was used to analyze relationships between bAA levels, genitalia,



behavior, and latency between male removal and sacrifice. All data in the text are presented as means  $\pm$  SEM.

## Results

In this study, the GSI was  $1.95 \pm 0.28$  for females (n=50),  $1.31 \pm 0.14$  for males (n=11), and  $1.37 \pm 0.27$  for sex changers (n=13). Females had a larger range of values (0.4-11.1) than either males (0.5-1.9) or sex changers (0.7-4.3). The average GSI was similar across groups (Kruskal Wallis test,  $H=3.52$ ,  $p=0.74$ , Fig. 22).

Of the groups that were planned for sacrifice within 30 min after male removal, 43.3% doubled their baseline displacement behavior at 10 min, 20% doubled at 20 min, 10% doubled at 30 min, and 26% did not double by the end of 30 min. The baseline displacement behavior for these groups and control females was not significantly different ( $F_{4,44}=2.49$ ,  $p>0.06$ ; Fig. 23)

There were no significant differences in bAA for any of the female groups within thirty minutes of male removal, whether they doubled their baseline behavior or not ( $F_{4,44}=7.22$ ,  $p=0.58$ ; Fig. 24). Further, there was no correlation between bAA and baseline or final displacement behavior or increases in displacement behavior among dominant females (all  $R^2<0.01$ ,  $p>0.81$ ; Fig. 25).

## Discussion

The results of this study do not support the idea that a down-regulation in whole bAA leads to increases in aggressive behavior, as the aggressive behavior doubled in groups with no

correlated differences in bAA levels. This was clear from the results, but there were other minor differences between this study and that of Chapter 4 that should be discussed.

Sex changers had a GSI similar to that of males, as in Chapter 4. The GSI values for males and sex changers of this chapter and Chapter 4 were in the range of those found in field fish by St. Mary (1993). The GSI values for female fish in this study were slightly lower than those in Chapter 4. The range of female GSI in this study encompasses the values seen in the previous study, and I know the fish were reproductive because both eggs and sperm were being made for the sex changer to fertilize eggs. It may be that in this study more female fish had laid their eggs prior to being sacrificed and having their GSI measured. This difference in GSI might also be explained by seasonal differences (see below). In a review by Miller (1984) of 29 gobiid species, female GSI ranged from 12-25 and males had lower GSIs ranging from 0.3-3.6, suggesting that *L. dalli* females have lower GSI than other gobiid species.

There are also differences between levels of brain aromatase activity in the control females between this study and Chapter 4. These may be due to differences between the assays or seasonal differences. This study ran from July 2003 to January 2004, while the study from Chapter 4 ran from February to March 2003. Seasonal differences have been observed in gonadal aromatase activity in channel catfish (Trant *et al.*, 1997) and black porgy (Lee *et al.*, 2000, 2001), and increased estrogens from the gonads may have effects on bAA. Further, brain aromatase activity was found to be different in summer than winter in perch (Noaksson *et al.*, 2003) and in goldfish (Pasmanik and Callard, 1988). In the black porgy, forebrain aromatase activity was found to be significantly higher in January than other times of the year and midbrain and hindbrain levels were highest in January and February (Lee *et al.*, 2000). Similarly, European sea bass had their highest levels of aromatase activity in the brain in January and

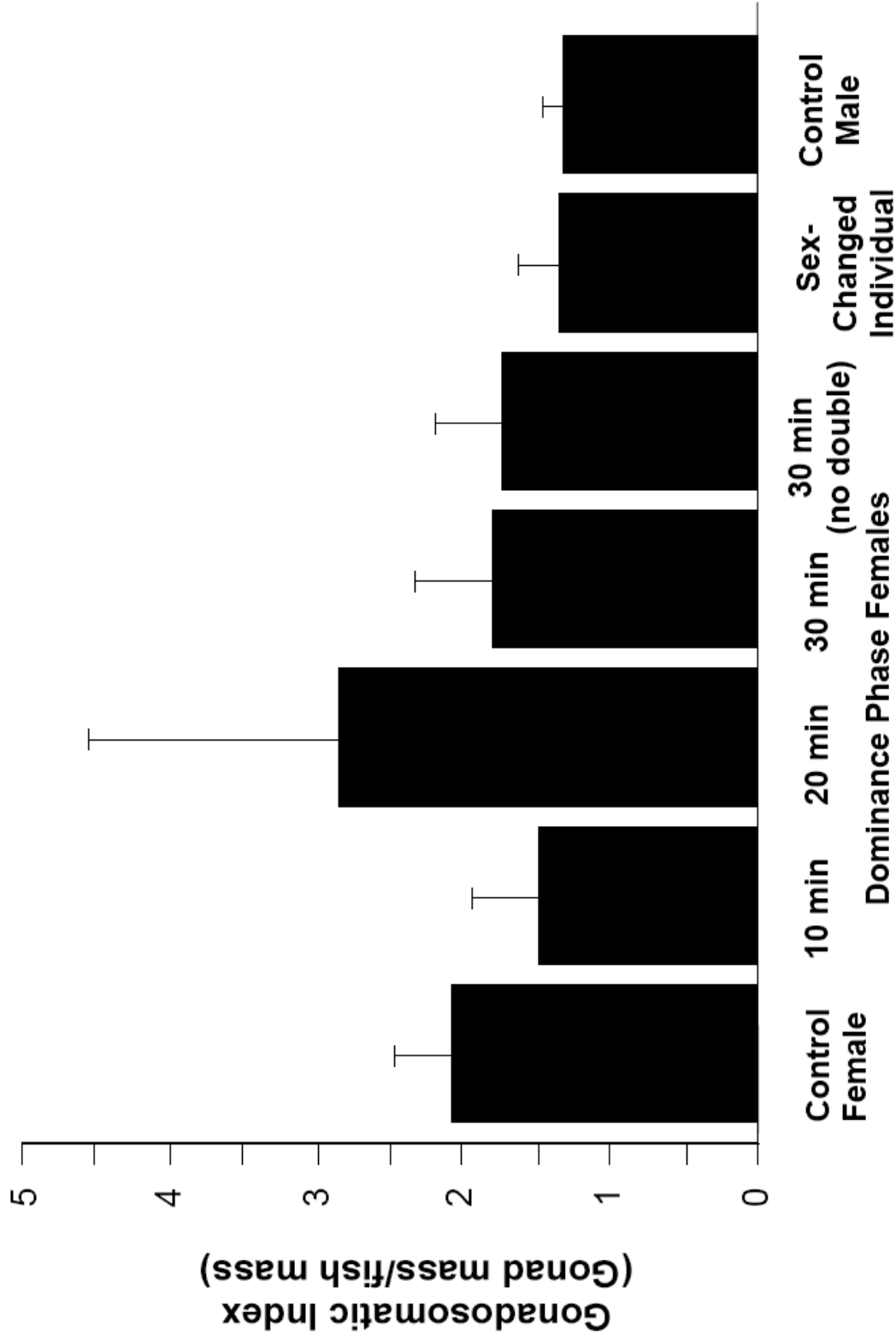


Figure 22: The gonadosomatic index (GSI, gonad mass/body mass x 100) for control females (n=19), females that doubled their baseline displacements 10-min (n=13), 20-min (n=6), or 30-min (n=4) after male removal, those females that went 30-min without doubling their displacements (n=8), control males (n=11), and sex changers (n=13). GSI was not statistically different between groups (Kruskal Wallis test,  $H=3.52$ ,  $p=0.74$ ).

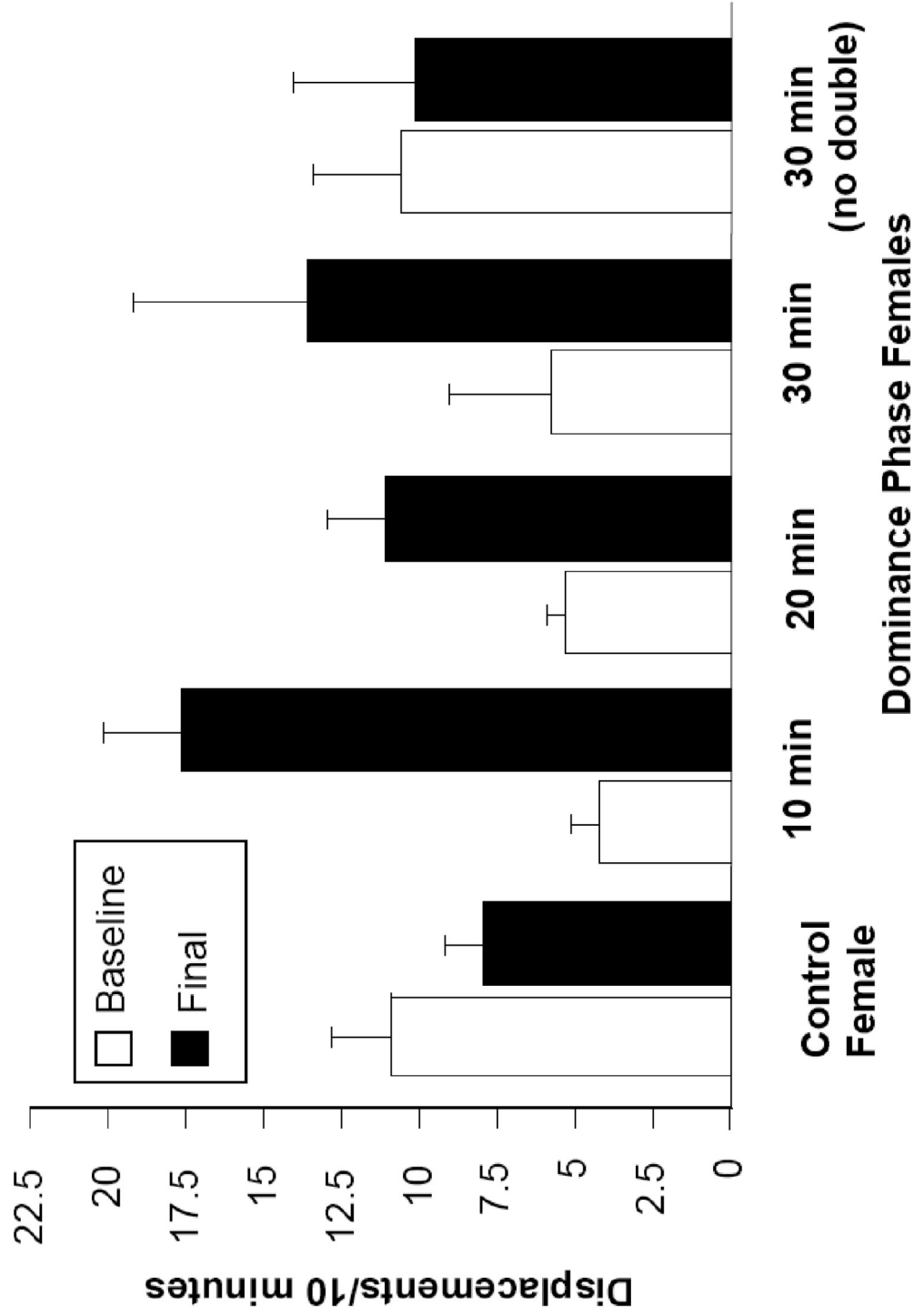


Figure 23: Displacement behavior on Day 4 (prior to male removal) showed no statistical difference in average daily displacements (baseline frequency, white bars) between the largest females. On Day 5, the male was removed from all but the control group (black bars). Day 5 control females showed lower levels of displacements and those fish that did not double their displacements by 30 min showed similar displacement behavior to their original baseline levels. (Control females n=19, 10 min. n=13, 20 min. n=6, 30 min. n=3, 30 min with no behavior doubling n=8)

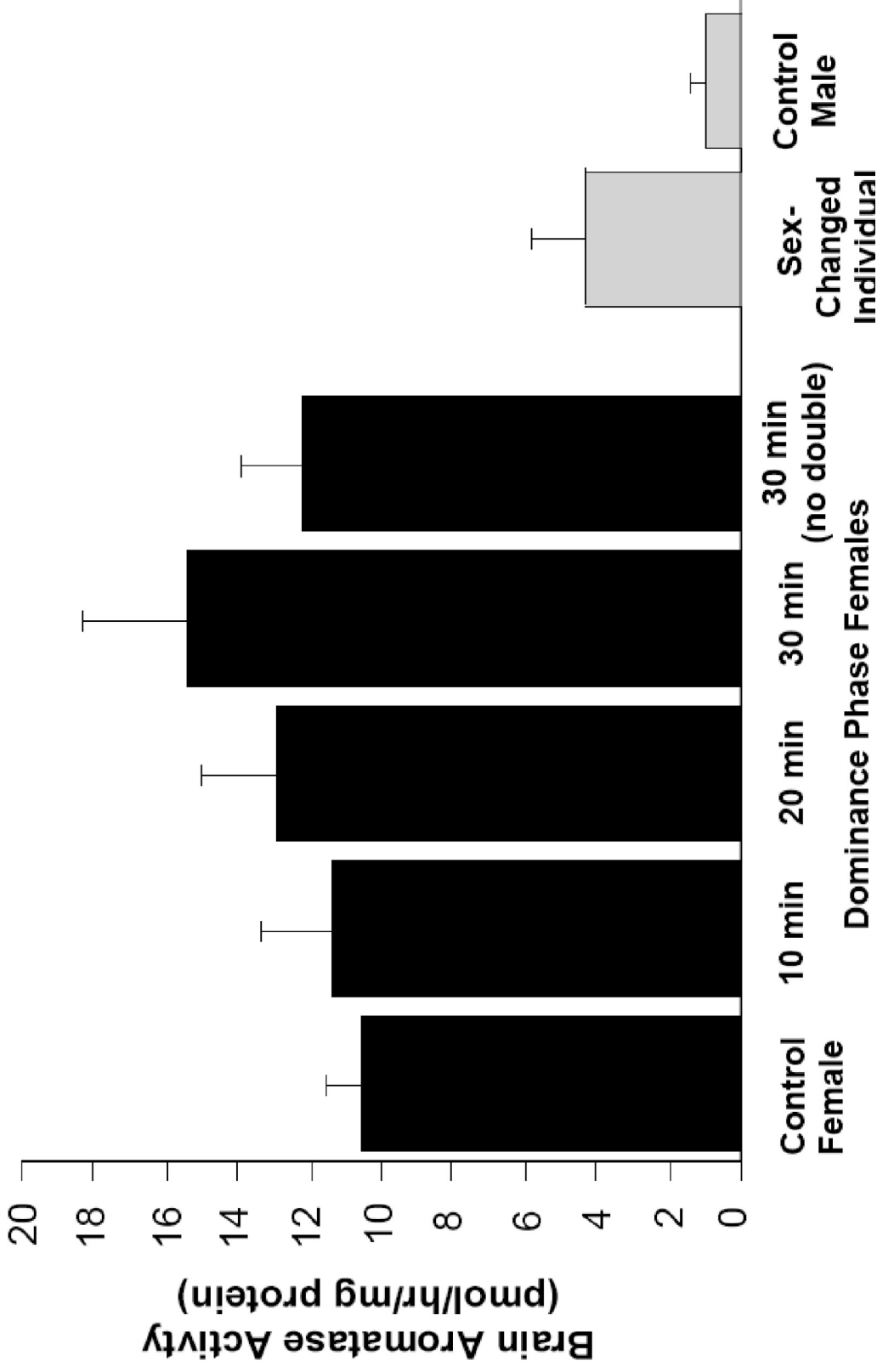


Figure 24: Brain aromatase activity for control females and females sacrificed at 10, 20 and 30 min after male removal. The females sacrificed showed a doubling from their baseline displacement behavior in the 10-min period just prior to sacrifice. The last black bar represents the females that were sacrificed at 30 minutes but did not double their displacement behavior in any of the three 10-min periods. The last two groups, sex-changed individuals and control males, are in grey because the samples had melted on the way to Belgium and levels may be artificially low. (Control females n=19, 10 min. n=13, 20 min.

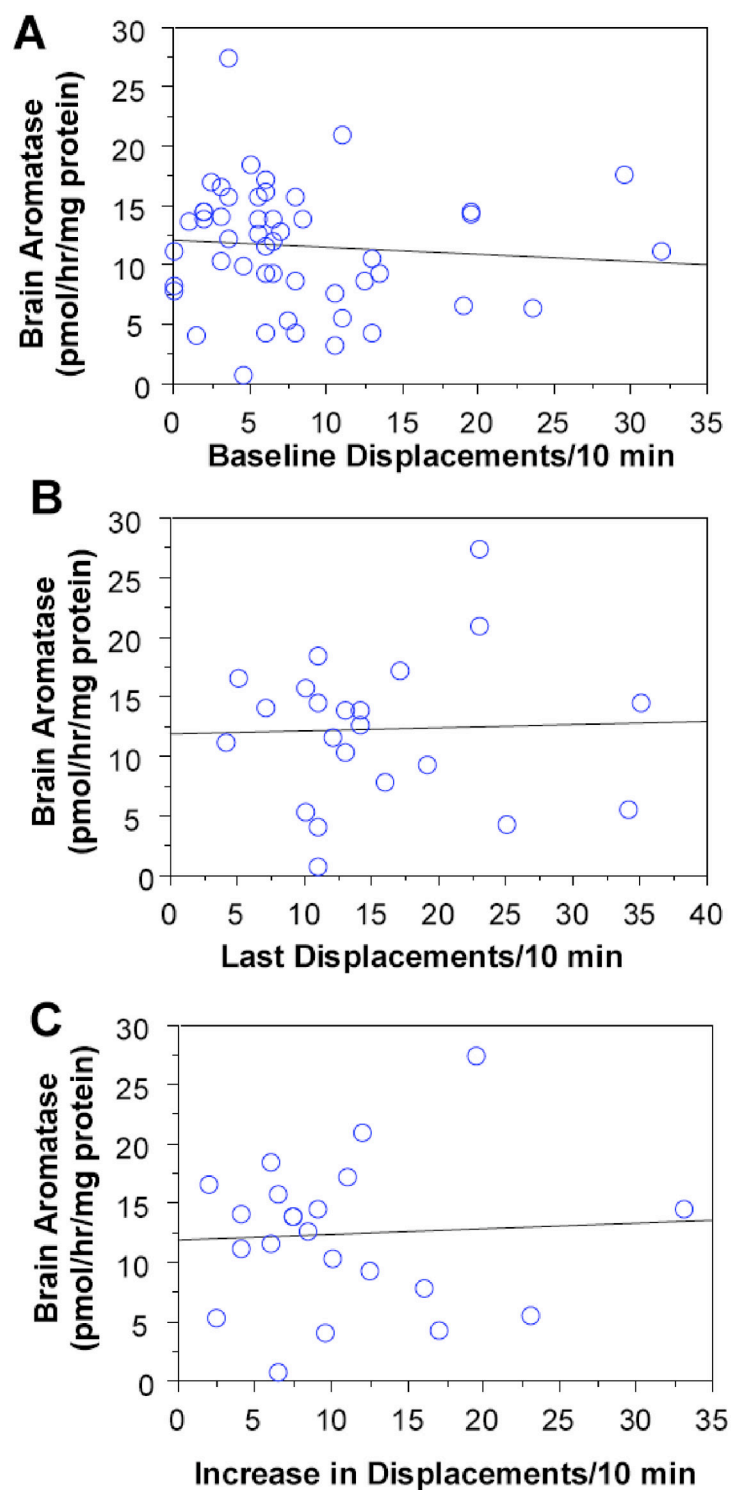


Figure 25: Regressions of brain aromatase activity and baseline displacements (A), final displacements (B), and the increase in displacements (final-baseline displacements, C). Graphs A and B include all females (n=49) and Graph C includes all females that showed increases in behavior (n=32). There was no correlation between brain aromatase activity and any of these parameters.

February (Gonzalez and Piferrer, 2003), although this is their time of spawning. January and February are not typically the spawning time in the wild of bluebanded gobies (Wiley, 1976; Behrents, 1983), even though in my controlled lab conditions the gobies continue to spawn.

Whether there were seasonal differences between this and the previous study or not, the results of this study do not support the idea that a down-regulation in aromatase activity leads to rapid increases in aggressive behavior, as the aggressive behavior doubled in groups with no correlated change in bAA levels. It is possible that the changes in bAA may be localized such that the whole brain assay was not sensitive enough to detect differences within hours that might still correlate with increased levels of aggression. However, this seems unlikely because the bAA was higher in later sampling time points rather than lower. Behavioral observations of fish implanted with an aromatase inhibitor such as fadrozole could be used to rule out this possibility.

Considering the correlation between bAA and aggressive behavior in Chapter 4, one alternative hypothesis is that the increase in aggressive behavior of the dominant fish as it begins to change sex is what affects the decrease in bAA. This “self-stimulation” occurs when an individual’s behavioral response to an external cue causes a change in the hormone levels rather than the external cue itself (Cheng, 2003). This process of an individual’s own behavior causing changes in that individual’s plasma hormone levels is seen in both lizards and birds (Cheng *et al.*, 1998; Yang and Wilczynski, 2002; Cheng, 2003). The correlation between increased aggression and bAA found in Chapter 4 may be another example of “self-stimulation,” but instead of detecting a change in the plasma hormones (an output), I detected changes at a local source: a change in brain steroid metabolism.

A further alternative hypothesis is that decreases in bAA and increases in aggressive behavior of dominant females are happening in parallel and do not have a direct connection. This

could involve an individual independent signal that turns on both pathways (decreasing bAA and increasing aggressive behavior) or different signals linked in the fish's life history that each trigger a different pathway.

The evidence from this study clearly refutes the hypothesis that decreasing whole brain aromatase activity leads to increases in aggressive behavior in dominant females at an early stage of sex change.



## CHAPTER 6: GENERAL DISCUSSION

The goal of this study was to understand how changes in social interactions affect the brain, steroid modulation, behavior, and morphology. The removal of a male from a social group in *Lythrypnus dalli* results in a suite of changes in the behavior, morphology, and physiology of the dominant female fish both in the laboratory and under field conditions. Chapter 2 established parallels between the process of socially-mediated changes in behavior, anatomy, and physiology in the laboratory and in the field. Chapter 3 demonstrated that cells containing isotocin-like (IST) peptide are different in location from vasotocin (AVT) containing cells and decreased in cell number during sex change. During this same change, AVT cells increase in size (Reavis and Grober, 1999). Chapter 4 shows that while gonadal aromatase activity (gAA) remained similar throughout sex change, during early sex change both aggressive behavior and brain aromatase activity (bAA) differed from control levels. Further, increases in aggressive behavior and levels of brain aromatase activity (bAA) were negatively correlated in the dominant female following male removal. Chapter 5 shows that although bAA and increased aggressive behavior were correlated hours after male removal (Chapter 4), earlier in the process the increases in behavior occurred before the changes in bAA.

Some important general concepts emerge from this dissertation. First, there can be a great deal of neuroendocrine and morphological within-sex variation in both males and females despite similarities in within-sex external morphology and reproductive behavior. Second, social interactions can modulate steroid metabolism and behavior on a short time scale. Third, local changes in steroid metabolism can occur in the brain independent of gonadal modulation of steroids.

## Variation in males

Sex-changed fish continue to change after fertilizing eggs as a male. The remaining ovarian tissue in new males (Chapters 1 and 3) and the high levels of gAA in sex-changers (Chapter 4) suggest that the decrease in gAA during natural sex change is a longer-term change than the initial decrease in the brain. In terms of what may regulate these longer-term changes, Gardner *et al.* (2003) have suggested that a gonadotropin signal sent from the brain, the *Sox-5* gene, and PPAR/RXR heterodimers may down-regulate gonadal aromatase expression through known actions and binding sites present on the aromatase gene of some sex changers. *Sox-5* is an SRY-type high-mobility-group box transcription factor that is involved in expressing I $\kappa$ B $\beta$ , a protein that is highly expressed in developing male gonads (Budde *et al.*, 2002). PPAR/RXR is a heterodimer of retinoid X receptor (RXR) and peroxisome proliferator-activated receptors (PPAR), nuclear receptor ligands that inhibit aromatase in human granulosa cancer cells (Rubin *et al.*, 2002). Through the regulation of aromatase, each of these could play a role in the build up of testicular tissue and the breakdown of ovarian tissue by modulating the levels of androgens and estrogens.

The modulation of androgen and estrogen levels may also affect longer-term changes in the brain itself, as newly sex-changed *L. dalli* still had higher AA than established males. Based on promoter regions in the aromatase genes of other sex-changing fish, Gardener *et al.* (2003) suggest *Sox-5*, *Sox-9*, *WT1*, NF- $\kappa$ B, PPAR/RXR, androgens, and progesterone as possible down-regulators of brain aromatase. *Sox-9* is one of the earliest markers within the pre-Sertoli cells of the developing testis, and overexpression of *Sox-9* causes male development of an XX individual (Huang *et al.*, 1999; Morrish and Sinclair, 2002). *WT1*, Wilms' tumor suppressor gene,

has a splice variant, +KTS, that is an important regulator for SRY in the sex determination pathway (Hammes *et al.*, 2001). NF- $\kappa$ B, nuclear factor- $\kappa$ B, is a rapidly regulated transcription factor involved in the apoptosis of germ cells (Baldwin, 1996; Pentikainen *et al.*, 2002). These may have effects on brain aromatase that future studies will determine.

In addition to the brain and gonad, the genital papilla length:width ratio for males is even higher than for sex-changers (Fig. 17). Based on the work of Carlisle *et al.* (2000), this difference in the genitalia could be due to 11-KT levels, suggesting that 11-KT levels in established or parenting males are even greater than those of newly sex-changed individuals, perhaps even playing a role in territorial behavior or parenting. 11-KT has been associated with territoriality in *S. viride* (Cardwell and Liley, 1991a) and also with established dominant male morphs in a variety of species (*e.g.* Nakamura *et al.*, 1989; Brantley *et al.*, 1993; Borg, 1994; Oliveira *et al.*, 1996, 2001a,b,c), so the idea of 11-KT being associated with dominance and territoriality is not a new one. However, in *L. dalli*, the change in genitalia is probably the result of other factors associated with dominance or the sex change process since there was no correlation between 11-KT levels and genital papilla length:width ratio (Chapter 2) and peripheral 11-KT has been shown not to rise during the sex change process (Rodgers *et al.*, unpublished), a time when it is known that genital papilla length:width ratios increase.

The differences in brain and morphology observed between established males and newly sex-changed individuals, including genital papilla ratios, bAA and gAA, are possible reasons why Reavis and Grober (1999) observed a longer time required for males to change back to female, even though the process is possible. In a study of the both-ways sex changer *Trimma okinawae*, male to female (M→F) transformations took longer than female to male (F→M) transformations (not statistically different, but an average of 9 vs. 5 days, respectively).

However, F→M→F transformations took longer than the two added together. The initial F→M transition was the same length, but the following M→F took 18 days instead of 9 (Grober and Sunobe, 1996). This suggests there is a mechanistic hurdle to going back after a trajectory in one direction has started. The behavioral model of Reavis and Grober (1999) could be used to replace the male at different stages of sex change to determine exactly when this mechanistic hurdle occurs relative to behavioral changes (Fig. 3). A similar mechanistic hurdle is present in other fish with socially-regulated reproduction. *Haplochromis burtoni* individuals that have changed from a nonreproductive male to a reproductive male can change back to the nonreproductive form and its behavior changes rapidly. The corresponding GnRH expression changes quickly in the initial direction, but lags in returning to its previous condition (White *et al.*, 2002). It would be interesting to see if aromatase activity has a similar lag when *L. dalli* change in the reverse direction from male to female.

Like *H. burtoni*, the aggressive behavior of *L. dalli* and the sex-specific mating behavior of *T. okinawae* change within minutes to hours of a social trigger. In *L. dalli*, the number of cells containing IST peptide was greater in females than males. However, the number appeared to decrease progressively over the sex change process rather than on the time course of behavioral changes, suggesting that IST is not involved in the early behavioral changes. Likewise, although the changes in steroid metabolism as measured by aromatase activity correlated with early changes in behavior, the behavior changed before different levels of bAA could be detected. Therefore bAA may instead result from the changing behavior. Further, similar and lower levels of aromatase activity are seen in small subordinate fish that are not changing sex, suggesting that a down-regulation in aromatase activity alone in the brain is not sufficient to cause sex change in these fish, even if it is correlates with early stages of sex change in the dominant females.

### **Social effects on brain aromatase activity and neuroendocrine modulation**

BAA in the dominant female appears to change quickly as a result of changing social circumstances. The only other research to document changes in bAA within hours of a social stimulus is a study with ring doves, *Streptopelia* sp. (Dudley *et al.*, 1984). Male ring doves presented with females had higher bAA in the preoptic area within 24 but not at 4 hr compared to isolated males, so the effect was not as fast as the average time for bAA to change in Chapter 4. It is hard to tease apart whether the isolation of ring dove males itself had effects on aromatase. It is also hard to determine if the bAA in the preoptic area had changed faster than 24 hr because only 4, 24, and 120 hr time points were examined. Although the authors stated that male behavior changes in the presence of females, they did not quantify the relationship between behavior and aromatase activity. The time scale of the change led the authors to suggest that known surges of plasma luteinizing hormone surges following paring with a female would cause known surges in plasma testosterone, which would then increase the bAA. Still, this is another example of vertebrate bAA changing within hours a social change.

Following the social change in *L. dalli*, there are many possible ramifications of the dominant female's decreased bAA in the short-term and the longer-term decrease in gAA. One possible outcome of lower AA proposed here is an increase in 11-KT levels (Fig. 20). AA inhibition by implanted fadrozole resulted in increased serum 11-KT and sex change in honeycomb grouper, *E. merra* (Bhandari *et al.*, 2003b, 2004). Further, exogenous administration of the steroid 11-ketotestosterone in *L. dalli* causes changes in the genitalia and gonads and development of the accessory gonadal structure both in socially isolated fish and in small female

fish paired with a larger female fish (Carlisle *et al.*, 2000; Carlisle, 2001; Rodgers *et al.*, unpublished). The high gAA in sex-changed fish suggests that perhaps peripheral 11-KT is not playing a large role in the process of natural sex change, but may be playing a role following sex change. This idea is supported by work from Nakamura *et al.* (1989) showing that with changes in the expression of gonadal aromatase, estradiol dropped rapidly while 11-KT was only higher in established males and did not increase significantly during the process of sex change in the saddleback wrasse, *Thalassoma duperrey*. The idea is further supported by preliminary work from Rodgers *et al.* (unpublished) that peripheral 11-KT is not rising in dominant *L. dalli* females during the sex change process.

Although 11-KT was correlated with the displacement behavior of sex-changed fish in Chapter 2, studies with 11-KT in *L. dalli* and other sex changing fish suggest that 11-KT alone is not sufficient to cause changes in dominance behavior. 11-KT implants in the smaller fish of *L. dalli* pairs did not increase aggressive displacements or courtship jerk swim behavior (Rodgers *et al.*, unpublished data). Sneaker males of the peacock blenny, *Salarias pavo*, did not increase male courtship or aggressive behavior with 11-KT implants, although they did show less female-typical courtship behavior (Oliveira *et al.*, 2001d). Similarly, 11-KT implants in non-territorial male and in female bluehead wrasse, *T. bifasciatum*, increased the expression of courtship behavior, but only when the dominant territorial male was not present (Semsar *et al.*, 2001; Semsar and Godwin, 2004). This suggests 11-KT could increase the likelihood of dominant male-typical behavior, but that the behavior is expressed only by the female or non-territorial male in permissive situations (*e.g.* when the dominant male is elsewhere or if the male is removed). As this is the situation for dominant females once the male has been removed, the

maintenance of male-typical and dominant behavior could be affected by local increases in 11-KT in the brain, even if 11-KT has not increased peripherally.

Another consequence of decreasing AA is decreased levels of estrogen and potentially rising levels of testosterone. Aromatase inhibition in *E. merra* by fadrozole resulted in lowered levels of estrogen and higher levels of testosterone (Bhandari *et al.*, 2004). A decrease in brain estrogen levels could explain the lower levels of cells in the preoptic area containing isotocin peptide. Steroids are known to have effects on neuropeptides and receptors such as those found in the vasopressin/oxytocin system (reviewed in Insel *et al.*, 1997; Goodson and Bass, 2001). For instance, estrogen can increase oxytocin receptors and progesterone can rapidly (within 30 min) increase the binding affinity of oxytocin receptors and facilitate lordosis, a female sexual behavior seen in rats (Schumacher *et al.*, 1990). In ovariectomized sheep, treatment with progesterone or estrogen alone increases oxytocin peptide mRNA expression in the periventricular nucleus, the medial preoptic area, and the bed nucleus of the stria terminalis (Broad *et al.*, 1993). In quail, estrogen given to males or an aromatase inhibitor given to females during development (day 9 of incubation) organizes the brain such that vasotocin-immunoreactive fibers in the medial preoptic nucleus are present as they would be in the opposite sex, despite a similar later hormone environment through gonadectomy and testosterone implants as adults (Panzica *et al.*, 1998). The females that had been treated with aromatase inhibitor during development even attempted copulations with females as adults (Panzica *et al.*, 1998). The vasotocin and isotocin neuropeptide sex differences seen in *L. dalli* may be affected by the differences in AA in the brain by way of decreasing levels of estrogen and rising levels of testosterone in sex changers.

Decreasing levels of estrogen have also been suggested as a key component of female to male sex change (Nakamura *et al.*, 1989, 2003; Higa *et al.*, 2003). Further, a purportedly unaromatizable form of testosterone, 17-methyltestosterone, alone and fadrozole alone caused genetic females to change to males during development in Japanese flounder, *Paralichthys olivaceus* (Kitano *et al.*, 2000), supporting the idea that decreasing estradiol and rising testosterone levels may be important in the sexual differentiation of fish.

### **Aromatase activity in fish and other vertebrates**

Steroid levels in fish are modulated by two different genes for aromatase: *cyp a*, found primarily in the gonad, and *cyp b* found primarily in the brain (*cyp a* and *cyp b* in goldfish, Callard and Tchoudakova, 1997, and zebrafish, Kishida and Callard, 2001; also called *p450aromA* and *p450aromb* by Kobayashi *et al.*, 2004, and *CYP19A1* and *CYP19A2* by Gardner *et al.*, 2003). In contrast, only one gene, *CYP19*, has been identified for human aromatase, and tissue specific promoters control its distribution, at least in part (Simpson *et al.*, 1994; Lephart, 1996; Simpson, 2003). Another potential difference in aromatase expression between humans and fish was discovered by Forlano *et al.* (2001) in the midshipman fish, *Porichthys notatus*. In *P. notatus*, aromatase expression tends to be most active in the glia (Forlano *et al.*, 2001). This may be similar to humans, where neurosteroids are “particularly but not exclusively synthesized in myelinating glial cells,” but comparative mammalian evidence suggests aromatase is located more in the neurons in mammals (Lephart, 1996; Baulieu, 1998). Despite these potential differences, all vertebrates appear to have the same aromatase enzyme and phylogenetic information, suggesting there are similar distributions of aromatase. In vertebrates, the anterior



forebrain areas known to control reproduction and sex behavior, such as the medial preoptic area, tend to have high aromatase levels and this distribution of aromatase activity seems to be conserved (Lephart, 1996; Balthazart and Ball, 1998).

The methods used to assess aromatase distribution and potential function can be very important because different methods can sometimes show similar patterns, but because of regulation at different steps they can also show different patterns. Forlano *et al.* (2001) found aromatase mRNA and aromatase-ir cells throughout the brain in *P. notatus*, with both mRNA and aromatase-ir cell density being high in the anterior regions of the brain, particularly in the telencephalon, and especially in the preoptic area within that region, as seen in most vertebrates. In a similar pattern, aromatase activity is also high throughout the brain in *P. notatus*, with the highest levels being anterior, the lowest being posterior, and high levels in particular in dissected pieces of telencephalon that contained most of the preoptic area (Schlinger *et al.*, 1999). However, this correlation between mRNA, the enzyme levels, and aromatase activity is not always the case. An example of this is seen in *Rana esculenta*, the edible frog, which displayed little increase in aromatase enzyme over the seasons, but large increases in estrogen production (Guerriero *et al.*, 2000). This suggests that there are post-translational mechanisms for changing aromatase activity. I investigated aromatase activity in my experiments because it is closer to the actual modulation of steroids than aromatase mRNA or protein levels. Aromatase activity can be post-translationally affected by changes in cofactors, accessory proteins, and nitric oxide concentrations (Bird and Conley, 2002; Balthazart *et al.*, 2003a,b). In comparing *L. dalli* control females versus dominance females hours after male removal, although it is possible that differences in aromatase activity could be due to differences in aromatase enzyme levels, it seems more likely that post-translational modifications were responsible for the nearly 50% drop

in activity levels of the enzyme that occurred not just in one area but in the entire brain. To verify a post-translational effect, levels of aromatase mRNA and enzyme levels within the brain between control females and recently sex-changed individuals should be compared. Both the gene regulators and the possible post-translational difference in bAA in *L. dalli* are potentially important in human research because investigations examining the mechanisms of local aromatase down-regulation may provide insight into ways that aromatase may be shut down quickly with fewer adverse effects than aromatase inhibitors currently used in treating breast cancer (Ragaz, 2001; Goss *et al.*, 2003).

### **Local brain changes independent of the gonads**

Changes in brain aromatase activity are not the only rapid changes of steroid metabolism observed in the brain. Soma and colleagues (2004) also have found that the enzyme 3 $\beta$ -hydroxysteroid dehydrogenase decreases in the adult female zebra finch brain within 10 min following acute restraint stress, such that less dehydroepiandrosterone (DHEA) is metabolized into androstenedione, the precursor for estrone and testosterone. Interestingly, DHEA promotes aggressive song in song sparrows during the non-breeding season (Soma *et al.*, 2002), a period when castration does not influence aggression (Wingfield, 1994) and aromatase inhibitors decrease aggression (Soma *et al.*, 2000a, b). Local steroid metabolism within the brain appears to be very important among vertebrates in regulating aggressive, reproductive, and parental behavior (*e.g.* Soma *et al.*, 2003, 2004; Trainor *et al.*, 2003, 2004), and is perhaps overlooked in many cases because it is easier to manipulate peripheral levels of steroids and look at the effects than to measure local changes in the brain, particularly on a short time scale. Because the brain

can modulate its own local steroid levels independent of the gonad, the work in this dissertation combined with recent literature suggests that local steroid metabolism in the brain merits more attention, particularly since steroids can have profound effects on behavior and brain morphology. For example, to activate sexual behavior in male, aromatase activity appears to play a critical role independent of the density of vasotocin-immunoreactive fiber tracts that were previously thought to be critically important for activation of male sexual behavior (Viglietti-Panzica *et al.*, 2001).

Brain steroid modulation independent of gonadal modulation could have profound effects on vertebrates in general. Local changes in brain steroid synthesis can serve as a way for the brain to avoid large unwanted effects of peripheral steroids from the gonads or modulate behavioral circuits with steroids when peripheral steroid levels are low. For species such as fish, a high level of bAA converts increased or high levels of testosterone into estradiol, preventing unwanted effects of testosterone on the brain. In other species, bAA may allow estradiol-mediated changes in behavior even when peripheral estradiol is low. Behavior often thought to be regulated by steroid hormones can be modulated independent of peripheral hormone levels, as seen in the sexual behavior of social animals such as primates (Wallen, 2001) and the aggressive behavior of animals, such as birds (Wingfield, 1994). In both of these cases, changes in brain steroid synthesis could serve as a mechanism for behavioral changes independent of peripheral steroid changes. Social modulation of these local steroid levels on a fast time scale may help to modulate and coordinate responses in brain circuitry for fast and appropriate social responses throughout vertebrate species.

In conclusion, the work in this dissertation shows changes in social interactions can cause profound changes in the brain, steroid synthesis, behavior, and morphology. This is one of the

few examples of a lab-derived result being tested and successfully working in the field. The parallels between laboratory and field results suggest changes in morphology and behavior observed in the laboratory are naturally relevant to this fish. Differences between the field and laboratory work, such as a lack of correlation between genitalia and 11-KT, inform the understanding of those laboratory results that may be relevant to the natural changes versus those that may simply be pharmacological effects or by-products of the laboratory environment. This dissertation work is the first demonstration of a sex difference in isotocin anatomy, a result that suggests homologous neuropeptides have a conserved sexually-dimorphic anatomy among vertebrates. In addition to there being profound sex differences in the brain (*e.g.* neuropeptides like isotocin), this work shows there can be profound brain differences within a sex (*e.g.* bAA and gAA). Further, bAA can change rapidly in response to a social cue independently of the gonad and this independent modulation may result from an individual's own behavior. Following male removal, the increase in the dominant female's aggressive behavior preceded the decrease in bAA, but negatively correlated with the bAA levels hours after male removal, suggesting the behavior might change the bAA levels. These results may have many implications for other species, particularly in responses to a changing social environment and brain regulation of steroid synthesis independent of the gonad.

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## APPENDIX: ACKNOWLEDGEMENTS FOR PUBLISHED PAPERS

The published paper titles and authors are listed at the start of each chapter. Parts of a published paper (Black, M. P. and Grober, M.S. 2003. Group sex, sex change, and parasitic males: Sexual strategies among the fishes and their neurobiological correlates. *Annual Review of Sex Research* 14:160-84) were modified and used in parts of the General Introduction and the General Discussion with permission from The Society for the Scientific Study of Sexuality. Acknowledgements for those who helped with published papers that have been modified into chapters of this dissertation are below:

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