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SEXUAL PLASTICITY IN A MARINE GOBY, *LYTHRYPNUS DALLI*: SOCIAL, ENDOCRINE, AND  
GENETIC INFLUENCES ON FUNCTIONAL SEX

by

EDMUND W RODGERS III

Under the direction of Dr. Matthew S Grober

ABSTRACT

Sex determination occurs early in development for most animals, at which time sex is fixed for life. Many teleost fishes, however, exhibit remarkable sexual plasticity throughout their life history, ranging from multiple morphs within a sex to functional adult sex reversal. To understand the development and evolution of adult sex reversal, I examined behavioral, endocrine, and genetic contributions to the regulation of functional sex in adult animals, using the bluebanded goby (*Lythrypnus dalli*) as an experimental model. This species was found to be equally capable of sexual transitions from female to male (protogyny) as from male to female (protandry). Throughout adult life, sexual phenotype is determined by social status, an emergent property of agonistic behavioral interactions that follows a relatively simple social convention: if dominant become or remain male, or if subordinate, become or remain female. The translation of social status into a change in sexual phenotype in the protogynous direction requires a rapid drop in circulating estrogens and an increase in the gonadal expression of a testis differentiating gene *dmrt1*. Steroid hormones do not play a significant role in modulating status, but the androgen 11-ketotestosterone does positively correlate with the expression of paternal behavior. Taken together, these findings suggest an evolutionary mechanism in sexually plastic species that has linked the conserved molecular cascades of sexual differentiation to a novel signal that varies over life history, social status, thereby allowing for lifelong phenotypic plasticity.

INDEX WORDS: Sexual plasticity, Protogyny, Protandry, Social dominance, *dmrt1*, Endocrine regulation, Paternal care

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EDMUND W RODGERS III

A Dissertation Submitted in Partial Fulfillment of the Requirements for the Degree of

Doctor of Philosophy

in the College of Arts and Sciences

Georgia State University  
2007

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## General Introduction

The sex of an individual animal is determined in one of two general ways, via genes (Genetic Sex Determination, GSD) or environment (ESD). Within ESD, the types of environmental signals and the time course over which they can act to determine the sex of an animal vary markedly. Those species where the functional sex (i.e. male or female) can be influenced outside of the realm of early development are deemed sexually plastic. One common form of sexual plasticity is hermaphroditism, wherein an individual can operate as both male and female at some point in their life history. Hermaphroditism comes in many forms, and can be selectively advantageous under a multitude of ecological conditions (Ghiselin, 1969), which helps to explain its prevalence in plant and animal clades, including mollusks, crustaceans, echinoderms, polychaete worms, and teleost fishes (Charnov, 1979; Ghiselin, 1969; Policansky, 1982; Grober and Rodgers 2007). A large group of advanced fishes, the teleosts, exhibit a stunning variety of sexually plastic phenotypes that range from variable expression of sex specific behavior within sexual morphs, to perhaps most strikingly, functional adult sex change (Devlin and Nagahama, 2002; Kuwamura and Nakashima, 1998). A flexible strategy presents challenges to the organism on a number of organizational levels that must be integrated to achieve a stable and advantageous evolutionary strategy. Using the bluebanded goby (*Lythrypnus dalli*) as a model system, this series of studies addresses these challenges at several biological levels, including behavioral regulation of sexual differentiation, endocrine regulation of somatic sex and possible influences on organizing sex specific behavior, and finally on the genetic machinery that manifests sex specific tissues. The goal is to produce a comprehensive and synthetic model concerning the evolution of sexual plasticity and the proximate regulation of that plasticity at various biological levels of organization.

### *Sequential hermaphroditism and the size advantage model*

Ghiselin (1969) developed a series of models that accounted for and described a number of conditions under which hermaphroditism would be selectively advantageous: low density model, size advantage model,

and gene dispersal model. The occurrence of sequential hermaphroditism, functioning as different sexes over the course of life history, but functioning as only one sex at a time, is best approximated by the size advantage model. The size advantage model states that sequential hermaphroditism would be selectively advantageous under conditions where an individual had greater reproductive success as one sex when young and small and as the opposite sex when older and larger, but noted that this model a) could incorporate, independent of body size, any traits that made it advantageous to be a member of a particular sex at a given stage of life history, and b) that an understanding of sequential hermaphroditism would extend beyond simple examinations of size (Ghiselin, 1969).

Extending the idea that differential reproduction between the sexes drives the evolution of sex change, Warner and colleagues (Warner, 1975; Warner et al., 1975) formalized Ghiselin's size advantage model, generating a mathematical model predicting the potential for, and direction of, adult sex reversal based on size-fecundity differences between the sexes in coral reef fishes. Given certain variables, such as female fecundity and population demography, an optimum size at sex change for a given species was predicted based on fecundity and age/size curves for each sex (Warner, 1975; Warner et al., 1975). If there were a single optimum size at sex change for a species, then selection would drive a mechanism that changed the sex of an animal at that size, likely through a genetic mechanism. This idea was falsified through empirical investigations that revealed that even within a species the average size at sex change varied between different populations (Shapiro, 1989). This demonstrated that it could not have been a genetically predetermined size, and, along with serious challenges to the assumptions of the model called into question its broad applicability (Shapiro, 1987; Shapiro, 1988). The essential question became, if age and size at sex change is not being genetically predetermined via a size based mechanism, how then was this information coded, and did this not violate the model?

The initial formulations of the size advantage model existed exclusively at the adaptive level of analysis (Sherman, 1988). The response to Shapiro's critiques was the first acknowledgement of the role of proximate issues; that relative size within the social group, rather than absolute size, determined size at sex change (Warner, 1988). This significant shift in the formalized model meant that the individual needed to assess local information in order to determine when sex change was warranted, and that selection could not be acting via a mechanism on the size of an individual itself. Interestingly, as an extension on the adaptive level, there appears to be a generalized trend across all sex changing species that the optimal time to change to the resource holding sex is about 72% of adult body size (Allsop and West, 2003), although the validity of this analysis has been challenged (Buston et al., 2004).

As exceptions to the model accrued over time, such as facultative sex change (Ross et al., 1990) and large protogynous animals foregoing sex change (Cole and Shapiro, 1995; Lutnesky, 1994), attempts were made to further extend the explanatory power of the model on the proximate level (Munoz and Warner, 2003). The expected reproductive success threshold model (ERST), the latest iteration of proximate level size advantage, requires an advanced calculus on the part of the individual, through which it compares its own reproductive success as both a male and female and decides from which sex it derives its greatest future reproductive success and allocates its reproductive energy accordingly. To assess its male potential it must calculate the fecundity of all other individuals within its social group, or home range depending on the species, in addition to the assumption that the sex of the other individuals will remain static. Given the advanced nature of these calculations, size advantage provides a compelling framework for answering the question of "why" sex change evolved, but does not provide a useful approach for understanding "how" it has evolved. Chapters 1, 3, and 4 will examine issues related to how sex change is regulated at the proximate level, while chapters 3 and 5 will touch on how it may have evolved.

### *Social regulation of sexual phenotype*

It has been known for some time that the sex of many sexually plastic species is regulated by interactions with the social environment (Fricke and Fricke, 1977; Robertson, 1972; Shapiro, 1979). The form of sexual plasticity expressed (e.g., protogyny, protandry, or bi-directionality) is influenced by ecological variables that structure the mating system and, by extension, the behavioral interactions among and between the sexes.

The responsiveness of sexual phenotype to variation in life history, environment, or behavior suggests the existence of latent plasticity inherent in the system but not likely to be seen unless the species is in the ‘appropriate’ habitat (Kuwamura et al., 2002). In protogynous sex change, two components are usually required: release from social inhibition, and stimulation from other reproductive individuals (Robertson, 1972; Ross et al., 1983; Shapiro, 1979). As a result of this two-component process, most species tend not to change sex in isolation (Carlisle et al., 2000; Cole and Shapiro, 1995). Protogynous fishes generally exist commonly in two forms, promiscuously breeding large colonies (Shapiro, 1979; Warner and Swearer, 1991) or small harem groups (Reavis and Grober, 1999; Robertson, 1972). In harem group living protogynous fishes, the general pattern of sex change is as follows: when the dominant male vacates the group, a new dominant individual emerges from agonistic interactions among the remaining females, who then transition to become the new male (Robertson, 1972).

In many bi-directional fishes, agonistic interactions determine which individual will change sex in same-sex pairs (Kuwamura et al., 2002; Nakashima et al., 1995; Sunobe and Nakazono, 1993), with the dominant being male (Kuwamura and Nakashima, 1998). In bi-directional species that are not subject to reproductive skew, this is not the case (Kuwamura et al., 1994; Nakashima et al., 1996), with several species following more closely to a growth-rate advantage model. Additionally, at least one species that is not subject to size fecundity skew, *Gobiodon histrio*, is also likely to use behavioral interactions to determine sex (Munday, 2002).

*L. dalli* has been described as a bi-directional sequential hermaphrodite (Reavis and Grober, 1999; St. Mary, 1993; St. Mary, 1994). These studies suggest that protogyny is robust and that protandry for this species is difficult, and that at the least it requires significantly more time to accomplish than protogyny (Reavis and Grober, 1999). Social groups in this species generally consist of one male and multiple females, although there is variation depending on habitat (personal observations). Upon exit of the male, the dominant female becomes the new male. Reavis and Grober (1999) describe the behavioral profiles accompanying protogynous sex change, that was verified under quasi-natural conditions (Black et al., 2005b). Generally, it involves an initial increase in aggression and a cessation of submission, followed closely by the expression of male specific courtship behavior. After this initial increase in rates of behavior, a quiescent period of low behavioral activity follows. Rates of behavior increase again as the new male initiates the first spawning.

Understanding in greater detail the social regulation, and the specific cues within that context, that regulate the expression of sexual phenotype is a main theme of several chapters (Chapters 1, 3, and 4), and is key to understanding not only proximate regulation of sexual phenotype but will providing insights into how these mechanisms came to evolve.

#### *Endocrine regulation of sexual phenotype*

Changes in reproductive behavior and/or the assumption of opposite sex behavior are not dependent on gonadal hormones (Godwin et al., 1996). But these decisions do direct physiological transformations that involve changes in sex specific steroid hormones (Frisch, 2004). With particular emphasis on protogyny, three major steroid hormones appear, in many species, to be involved in physiological, and possibly behavioral, transformation from the female to male sex: estrogens (E), testosterone (T), and 11-ketotestosterone (KT). A decline in estrogen at the onset is theorized to be a critical step in protogynous sex change (Nakamura et al., 2003). Estrogens are high during the female phase and decline rapidly over sex change in wrasses (Bhandari et al., 2003; Nakamura et al., 1989) and goby (Kroon and Liley, 2000). Treatment with aromatase inhibitors,

which decreases circulating estrogen, induces partial or complete protogynous sex change in the honeycomb grouper (Bhandari et al., 2005), the blackeye goby (Kroon and Liley, 2000), and the threespot wrasse (Higa et al., 2003). Suggestive of its role as an inhibitory step in the process, when E2 is administered with either KT or an aromatase inhibitor, it prevents protogynous sex change (Bhandari et al., 2005; Higa et al., 2003).

Aromatase protein also declines in the gonad during sex change in the Saddleback wrasse (Morrey et al., 1998).

The potent fish androgen KT (Borg, 1994) has been shown in several studies to increase over protogynous sex change (Bhandari et al., 2003; Cardwell and Liley, 1991; Nakamura et al., 1989). Exogenous KT has been shown to masculinize many traits in sexually plastic species, including color (Cardwell and Liley, 1991; Semsar and Godwin, 2003) and external genitalia (Carlisle et al., 2000), and to induce complete sex change (Higa et al., 2003; Kroon and Liley, 2000). KT has been shown to increase aggressive/ territorial behavior (citation) as well as induce male sexual behavior in female goldfish (Stacey and Kobayashi, 1996). KT is a potent masculinizing agent, but in several species of sexually plastic fishes it is either not different between males and females (Kroon et al., 2003; Rodgers et al., 2006) and/or low during sexual transitions (Johnson et al., 1998; Nakamura et al., 1989).

The role of testosterone, if any, in the sex change process varies by species and experimental manipulation. That is perhaps not surprising considering that both sexes of most species have detectable T (Frisch, 2004). In the saddleback wrasse, T does not vary over the course of sexual transition (Nakamura et al., 1989). Exogenous administration of T or a non-aromatizable form (17-alpha-methyltestosterone (MT)) induces sex change in some species (Yeh et al., 2003), but not in others (Kramer et al., 1988; Kroon and Liley, 2000). T has been shown to have opposite effects of promoting feminization and masculinization depending on the dosage (Yeh et al., 2003). The varied and contradictory effects of T administration have led to the theory that the primary mode of action for T is via its metabolites, E2 through aromatase and KT through the activity of 11-

betahydroxylase and 11-beta hydroxysteroid dehydrogenase (Kroon and Liley, 2000). Chapter 4 examines the interactions between steroid hormones, sexual phenotype and, behavior.

#### *Endocrine regulation of paternal care*

An important component of sex change in *L. dalli* is the transformation from a non-parenting phenotype (females) to a phenotype that invests heavily in care of eggs (males). Paternal care in *L. dalli* often co-occurs with, but at times is dissociated from, nest defense (e.g., when no clutches are present). Like many marine teleosts, *L. dalli* has a pelagic larval phase that begins immediately following hatching (Steele, 1997), and thus male parental care ends when eggs hatch. In many vertebrate species, androgens are elevated during some phases of the male reproductive cycle, namely courtship and aggressive behavior, and are depressed during others, such as paternal care (Oliveira et al., 2002; Wingfield, 1984; Wingfield et al., 1997). Although an inverse relationship between androgens and paternal care is present in many species (Ketterson and Nolan, 1994; Van Roo, 2004; Wynne-Edwards, 2001), it is not evident in all paternal species (Ros et al., 2004; Trainor and Marler, 2001; Trainor and Marler, 2002; Ziegler et al., 2004; Ziegler et al., 2000).

One common component of paternal care that may complicate the androgen relationship is aggression (Marler et al., 2003). Males of many species must actively defend a nest or territory while providing parental care. This necessity for aggressive behavior may preclude a decline in androgen levels during paternal care. Fish provide a unique opportunity to test these ideas, because many species exhibit high levels of paternal care that co-occur with vigorous nest/territory defense.

11-Ketotestosterone activates a suite of male typical behavioral and morphological traits in most fish species, including sex changing species and species exhibiting alternative male reproductive tactics (Oliveira, 2004). Interestingly, the territorial male phenotype in species exhibiting alternative reproductive tactics often provide exclusive care of offspring and as a rule have higher levels of KT than sneaker or satellite males (Brantley et al., 1993; Oliveira et al., 2001a). KT has been shown to be the androgen that mediates the



challenge response in fishes (Hirschenhauser et al., 2004). The role of androgens in paternal care in fishes has generally been addressed in two ways: by sampling endogenous androgens over the course of the mating cycle, or administering exogenous steroid hormones to parenting males. These studies have yielded mixed results. Many studies of endogenous androgens document a decline when a male fish is parenting, most often following an initial period of high androgens (Knapp et al., 1999; Oliveira et al., 2002; Pall et al., 2005; Pall et al., 2002a; Pankhurst, 1990; Sikkel, 1993; Specker and Kishida, 2000), while exogenous administration of androgens does not appear to inhibit paternal care to a substantial degree (Kindler et al., 1991; Pall et al., 2002b; Ros et al., 2004) and can facilitate parenting in the blue gourami (Kramer, 1972).

Over the course of naturally occurring sex change, *L. dalli* allows for comparisons of non-parenting females and inexperienced males to parenting males, and examination of whether hormone levels change as an animal transitions from a sex providing no care (female) and will readily engage in filial cannibalism, to the other that provides exclusive parental care (male). This species provides unique opportunities to examine how hormones may mediate potential trade-offs between aggression and parental care in species that must simultaneously display aggressive, courtship and parental behavior (Chapter 2).

### *Modular sex determination*

Sexual reproduction is nearly universal among animal species and at least two functionally distinct sexes are required for the success of such an approach. The sexes tend to be segregated by which individual produces large resource rich gametes or small, abundant, but resource-poor gametes. Switching between those two sexes and coordinating the suite of associated traits that characterize each of the sexes is an extraordinary adaptation. How sexually plastic species accomplish this at a molecular level, and how such systems evolved, are emerging questions in the field. Several alternative hypotheses exist to explain how this has occurred. Current dogma posits that sex change evolved as a series of unique adaptations to transform an ovary into a testis, in the case of protogyny or a testis into an ovary, in the case of protandry. An alternative is that sexually plastic species

evolved novel regulation of the conserved genetic mechanisms that produce ovaries and testes during the development of sexually fixed species. Once unconstrained by an early critical period during which lifelong sex is fixed, a regulatory element that varies over the life history of an animal, but is predictive of changes in reproductive opportunity, could provide an unbeatable evolutionary strategy. To test these alternative hypotheses, characterizing the role of well established sexual differentiation genes in a sexually plastic species is necessary. Recent work on a variety of sexually ‘fixed’ species has shown that the genetic factors that determine sex are highly variable (Zarkower, 2001), but the downstream mechanisms of sexual differentiation appear to be quite conserved (Marin and Baker, 1998; Morrish and Sinclair, 2002; Wilkins, 1995). Tests of this sort on sexually plastic species would allow us to gain novel insights into this fundamental question.

Recent work in *L. dalli* suggests that sex change evolved by entraining the conserved molecular pathways for sexual differentiation to a cue which varied over the life-history of an animal (variable signal model, Chapter 4) (Rodgers et al., 2007). While many upstream regulators of sex vary considerably, there is downstream convergence on a zinc-finger transcription factor involved in sexual differentiation from flies to humans; the DM family of transcription factors.

DM domain-containing genes are involved in sexual development from corals (Miller et al., 2003) to humans (Volff et al., 2003). *Dmrt1* knockout mice do not form testis properly (Raymond et al., 2000). Expression of *Dmrt1* or its recent duplicate *DMY* can induce sex-reversal in genetic females (Matsuda et al., 2007; Smith et al., 2003). *Dmrt1* orthologues share a highly conserved DM domain and varying degrees of similarity thereafter (Zhu et al., 2000) and are more closely assort together than with other DM paralogues (Volff et al., 2003). Characterizing this gene in the context of sex change in the bluebanded goby (Chapter 5) begins to illuminate some of the molecular machinery involved in sex change and may also reveal interesting insights into the evolution of sexual plasticity in metazoans.

## Chapter 1

Sex reversal in pairs of *Lythrypnus dalli*: behavioral and morphological changes

Rodgers, EW, Drane S, Grober, MS (2005) The Biological Bulletin; 208: 120-126

### **Abstract**

In the bluebanded goby, *Lythrypnus dalli*, reproductive success is primarily determined by functional sex, and functional sex is determined largely by rank in the dominance hierarchy. In most natural social groups of *L. dalli*, one male is at the apex of the hierarchy, and 1 to 7 females are lower in rank. When a male exits the group, a female ascends to the top of the hierarchy and becomes a male. We have examined this process in a simplified environment -- a pair of females -- which allows us to identify behavior associated with the formation of a dominance relationship and any other phenotypic changes associated with dominance and/or sex change. We found that *L. dalli* female pairs quickly and readily form stable dominance relationships, with the dominant fish changing sex into a male. This dominant animal also rapidly increased in body size and length of its dorsal fin. In summary, dominant *L. dalli* females change sex in this simplified environment, providing excellent opportunities to examine the early behavioral and morphological changes associated with dominance and sex change.

### Respective contributions

Miss Drane contributed many hours of observation in addition to aiding with the gonadal analysis. Dr Grober assisted in the editing of this MS and the statistical analysis.

## Introduction

Social context often determines how a given individual responds to behavioral stimuli, with individuals modifying their behavior based upon the behavior of the individuals around them. This is especially true in several species of protogynous (female to male) sex changing fish, where sex reversal is socially controlled. In these species, a unique pattern of behavior stimulates one individual to change sex while inhibiting others (Robertson, 1972; (Ross et al., 1983). Two social factors are thought to be the primary regulators of sex change: inhibition by males and stimulation from other females (Robertson, 1972; (Shapiro, 1979). Without sufficient social stimulation (i.e., in isolation), fish capable of sex reversal might not initiate sex change (Cole and Shapiro 1995; Carlisle et al., 2000). Size advantage (Warner et al., 1975) has been shown to contribute to the determination of which animal changes sex, but behavioral interactions are also critical determinants (Lutnesky 1996). Larger size often equates with increased success in aggressive encounters and therefore social dominance, providing a proximate mechanism for the size advantage hypothesis. In protogynous sex changers, the most reproductively significant resource that dominance affords is “maleness”; thus the reproductive payoff for dominance is extremely large and females would be highly motivated to increase their aggressive behavior in times of social instability (i.e. in the absence of a dominant male).

To date, the study of *L. dalli* has dealt exclusively with larger social groups (>3), and at present it is unknown if sex change can be induced in pairs of females. This study uses pairs of fish to closely examine changes associated with the acquisition of dominance, and potentially sex change. In a group environment, this is difficult to tease apart owing to multiple interactions between individuals in the group, whereas in pairs there are fewer social variables contributing to an animal’s behavior. We find that one of the pair quickly changes to as evidenced by male typical behavior and male reproductive morphology.

## Materials and Methods

We used four measures of “maleness” to determine if any of the fish changed sex: 1) display of male typical courting behavior, 2) male typical papilla ratio, defined as a greater than 1.6 length to width ratio (female ~1.0 (l/w), 3) the presence of an accessory gonadal structure (AGS), and 4) the presence of fertilized eggs, an unequivocal indication of functional sex change.

### *Subjects*

Thirty-two *L. dalli* females between 23-30 mm in length were used in the study. Sex was determined by examination of the external genital papilla (Behrents, 1983). *L. dalli* females have a papilla ratio (length / width) of approximately 1.0 whereas males have a ratio of approximately 2.0 (St. Mary, 1994); the largest ratio at the start of this experiment was 1.3. Fish with female typical papilla have gonads consisting of > 95% ovarian tissue (St. Mary 1994). During the experiments animals were housed in 33 L aquaria, each with an individual filter system (Marineland), at 68-70°F, fed twice daily using a commercially prepared diet (OSI Marine Labs) and kept on a 12h light/dark cycle. One group of fish were collected (CF & G permit # 803034-01) on Santa Catalina Island, California, in May 2002 (pre-breeding season), using an anaesthetic solution of quinaldine sulfate (Sigma Chemical) and hand-nets. A second group of fish was collected during the breeding season in late June 2002 using the same methods. Prior to the experiments, the animals were kept in 180 L holding tanks.

### *Experimental design*

The study is divided into two experiments. Experiment 1 consisted of the quantification of morphological traits and behavioral interactions between 8 pairs of individuals identified as females. External morphological data were collected prior to pairing (standard length, mass, and papilla ratio, described below). The pairs were observed until the presence of eyed eggs (a marker of fertilization), indicating functional sex change, or 14 days, an adequate amount of time for sex change in this species (Reavis and Grober 1999).

Experiment 2 was similar to Experiment 1, and was designed to confirm a novel observation in Experiment 1, that dominant fish exhibited disproportional growth of the longest rays of their dorsal fin, but was without extensive behavioral observation (n=8). In both experiments, the pairing and morphological measurements (with the exception of initial dorsal fin length) were identical (see below).

At the completion of the experiment, the animals were given an overdose of tricaine methanesulfate (MS 222), and their morphological characters were measured again. This included measurement of the longest dorsal ray (see Results). The animal's was placed in Bouin's fixative. The gonads were dissected out to examine internal morphology. Digital photographs of all the gonads were taken to aid in analysis.

During sex reversal, *L. dalli* undergoes gonadal reorganization (St. Mary, 1994), which in a number of sex changing gobies involves degeneration of ovarian tissue (Sudovy and Shapiro 1987) and generation of AGS and testicular tissue (Cole and Shapiro 1990). In *L. dalli*, the presence or absence of the AGS is the primary indicator of functional sex; males have a highly developed AGS and females have none. Visual inspection was used to assess the composition of reproductive tissue, i.e. presence of eggs and presence of AGS. Gonad allocation was verified using standard paraffin histology followed by Hemotoxylin and Eosin staining.

### *Pairing*

Animals were removed from the holding tank and anesthetized using tricaine methanesulfate (MS 222). Mass and standard length were recorded. Papilla ratios were measured, with digital images also taken. Paired animals were within 2 mm in standard length and 0.1 g of body mass. The fish were individually identified by their banding pattern, which did not appear to change nor have any effect on their behavior. Two animals were placed together into a 33 L tank with a single PVC tube to serve as the nest (St. Mary 1994).

### *Observations and Behavior*

Direct behavioral observations of each pair were conducted twice daily, once in the morning and once in the afternoon for 10 min per session. Data were recorded on paper then transferred to Excel™ spreadsheets.

The fish were given one day to acclimate before observations began. The observer recorded the number of approaches, displacements, jerks, bites, nips, solicitations, and tail-waggles. In addition, the observer noted which animal resided in the tube, if either animal was gravid, and whether or not eggs were present in the nest tube.

An approach is defined as a fish moving within 5 cm of the other fish. Movement away from the approaching fish is recorded as a displacement. Jerks are a male typical behavior used during courtship (Behrents, 1983), and involve a saltatory swim motion with movement laterally as well. Because jerks can be directed at the female or around the nest but not directed at a fish, a jerk towards a female is scored as both an approach and a jerk. Bites are aggressive interactions in which one fish bites the other after an approach. In contrast, nips occur after jerking to a female, with a male nipping the tail of the female. This behavior is also part of the courting process. Solicitations are produced by females when they move within 12 cm of the male in his line of view. Tail waggles are displayed by both sexes. They consist of a fish remaining stationary while moving its tail back and forth.

### *Statistics*

Morphological data were analyzed using parametric statistics. For traits that had ‘before’ and ‘after’ measures we used paired t-tests (standard length and fin length in experiment 2). For fin length in experiment 1, we used an unpaired t-test, examining at differences between males and females. We used a t-test to examine whether papilla morphology between new males and females was significantly different. The distributions of the frequencies of the various behaviors for newly sex-changed males and females did not meet the criteria for parametric statistics (e.g. normality, homogeneity of variance), thus we used non-parametric methods to examine group differences. All behavioral analyses were conducted with the Wilcoxon signed rank test. Values for mean and median were similar, indicating a symmetrical distribution. Simple linear regressions were employed to examine the relationship between known interdependent behaviors (e.g., approaches

regressed on displacements). Though the fish were size matched, we examined whether small differences in size predicted which individual would change sex using logistic analysis (SAS). Significance level in all cases was set at  $p < 0.05$ . Mean values  $\pm$  standard error are given. All analyses were carried out using the Statview 5.01™ (SAS Institute inc.) unless otherwise noted.

## Results

In all cases, the pairs readily established a stable dominance relationship, defined as one individual (the dominant) instigating the majority of encounters (Figure 1.1a) and exhibiting male courtship behavior (Figure 1.1b), while the other individual (the subordinate) is displaced as a result of the encounter (Figure 1.1c). In all cases the dominant individual initiated spawning behavior with the subordinate individual that resulted in a clutch of eggs. Four of the 8 clutches were verified as fertile; in the other 4 cases, the male consumed the clutch before verification. At the completion of the experiment, all dominants were found to have a male papilla ratio as well as an AGS (Figure 1.2, 1.3). Using our criteria, we demonstrated that the dominant individual in a pair of females changed sex from female to male.

### *Morphological Changes*

All fish initially had a female typical papillae ratio (mean  $\pm$  SEM:  $1.15 \pm 0.061$ ). There was no difference in papillae ratio at the start of the experiment ( $t = 0.732$ ,  $df = 14$ ,  $p > 0.05$ ) between those fish that became male (mean  $\pm$  SEM:  $1.15 \pm 0.102$ ) and those that remained female (mean  $\pm$  SEM:  $1.17 \pm 0.086$ ). After sex change there was a significant change to a male typical papilla in the fish that demonstrated male typical behavior ( $t = 8.64$ ,  $df = 14$ ,  $p < 0.0001$ ). Female papilla ratio remained the same,  $1.09 \pm 0.052$ , whereas in sex changers the ratio more than doubled to  $2.26 \pm 0.127$  (Figure 1.2).



All dominant animals exhibited unambiguous male typical gonad morphology that included the presence of sperm and an AGS. All subordinate individuals were gravid and eggs were clearly visible within the ovary upon inspection. These results were verified histologically (Figure 1.3).

Dominant individuals exhibited a significant change in standard length over the course of the experiment ( $p < 0.05$ ), while subordinates did not ( $p > 0.05$ ). We then examined the magnitude and direction of the change and found that dominants grew significantly more relative to subordinates (paired t-test,  $t = 4.528$ ,  $df = 15$ ,  $p = 0.0003$ ).

In experiment 1, we noted the dorsal fin of the dominant fish was elongated. Although we had not measured initial fin length, at the conclusion of the experiment we measured the longest dorsal ray. Dominants had a mean fin length of  $10.47 \pm 0.539$  mm while subordinates had a mean of  $5.5 \pm 0.269$  mm ( $t = 8.475$ ,  $df = 14$ ,  $p < 0.0001$ ). To control for dominant fish simply growing more, we used a ratio of fin length to body length. This ratio was significantly different, with subordinates having a smaller fin with an average of  $4.648 \pm 0.137$  fin lengths per body length and dominants having a ratio of  $2.64 \pm 0.143$  ( $t = 10.129$ ,  $df = 14$ ,  $p < 0.0001$ ).

Experiment 2 verified the marked change in fin length observed in the first experiment. Future dominants and subordinates did not have different fin lengths at the beginning of the experiment ( $t = 1.669$ ,  $df = 8$ ;  $p > 0.05$ ), but the dominant fish had a significantly longer fin at the end of the experiment ( $t = 4.091$ ,  $df = 8$ ;  $p = 0.0035$ ). Dominant individuals exhibited a pronounced lengthening of their longest dorsal ray, while many of the subordinates showed a fin shortening.

We attempted to minimize size as a major contributing factor; however small differences in size did exist between the paired animals. To assess whether these small differences in standard length were predictive of which animal changed sex, we ran a logistical regression. Our results suggest that the size differences present in this experiment were not a good predictor of which animal would become dominant (Wald  $\chi^2 = 0.034$ ,  $p = 0.8532$ ,  $df = 1$ ,  $\beta = -0.05006$ ).

## *Behavior*

Verification of egg fertility in Experiment 1 was difficult because of consumption of clutches by males (see below). Four of eight pairs completed sex change in less than 14 days, as determined by the presence of fertilized eggs. In the four remaining pairs, males exhibited all other sex typical characteristics, but consumed at least one complete clutch of eggs. Because fish that parented and fish that consumed their clutch displayed similar rates of behavior in all measures, we grouped them in the behavioral analysis.

A clear dominance relationship was evident from the first observational session and persisted unchanged throughout the experiment (Figure 1.1). The dominant fish produced male typical behavior and began courting the subordinate. The dominant fish instigated the vast majority of behavioral interactions, evidenced by high rates of approach behavior. Dominants displayed significantly higher rates of aggressive and courtship behavior (approaches and jerks,  $p < 0.05$ , Figure 1.1 a, b). Subordinates displayed significantly more submissive and female typical behavior, (displacements & solicitations,  $p < 0.05$ , Figure 1.1 c, d). All dominants jerked and subordinates generally did not jerk, although there were rare individual displays over the course of the experiment. Subordinate fish were displaced at high rates (9.138 per 20 min, Figure 1.1c) compared to dominants (0.062 per 20 min). There was a significant association between approaches and displacements, such that when a dominant approached, the subordinate was usually displaced ( $R^2 = 0.917$ ;  $p < 0.001$ ). The dominant guarded and/or resided in the tube for the majority of the time.

Other behavior occurred at a far lower frequency, including bites, nips, and tail waggles. Dominant individuals produced bites and nips. Tail-waggles were the one behavior that did not show an overall sex/dominance bias, with both dominants and subordinates expressing the behavior.

## Discussion

When two female bluebanded gobies were paired, in our experimental conditions, in all cases, one fish established dominance over the other, with that fish instigating the majority of encounters and winning nearly all of them. The dominant fish became male, exhibiting all behavioral and morphological characters associated with that sex. The subordinate fish remained female and exhibited all associated sex typical behavior and morphology.

In *L. dalli*, body size is sexually dimorphic with males being larger (Wiley, 1976). In our work dominant individuals grew more than subordinates and changed sex. The observed sexual dimorphism in growth rate is consistent with previous data from St. Mary (1994); however, we can not address whether the increase in growth is a result of sex change or dominance. Dominance rank has been shown to affect the growth rate of individuals within a hierarchy of sex changing clown fish (Buston, 2003). We also found that the dorsal fin was elongated in the dominant, sex changed fish. The dorsal fin was known to be sexually dimorphic (Wiley, 1976), but we did not expect the rapidity and magnitude of the changes we observed in the first experiment. Experiment 2 confirmed that the fin lengthened dramatically over the course of the experiment in the dominant individual. It is important to note that many of the subordinate fish showed shrinkage in their dorsal fin. Whether this is a natural process or the fin was shortened by the dominant through aggressive encounters is unknown. As with the change in growth rate, we cannot say whether fin elongation is caused by the sex change process, or whether its growth is modulated by dominance status. If the latter is the case, then this trait can potentially be used as an index of rank within a hierarchy.

Simplification of the social group to a pair of animals, as in our study, did reveal interesting insights that were not seen in previous studies using larger groups of fish: the interdependence of behavior between males and females. This is best illustrated in Figure 1b and d, where courtship behavior (solicitations and jerks) clearly show the same pattern over time. At this point, the precise nature of the interaction is unclear. Our

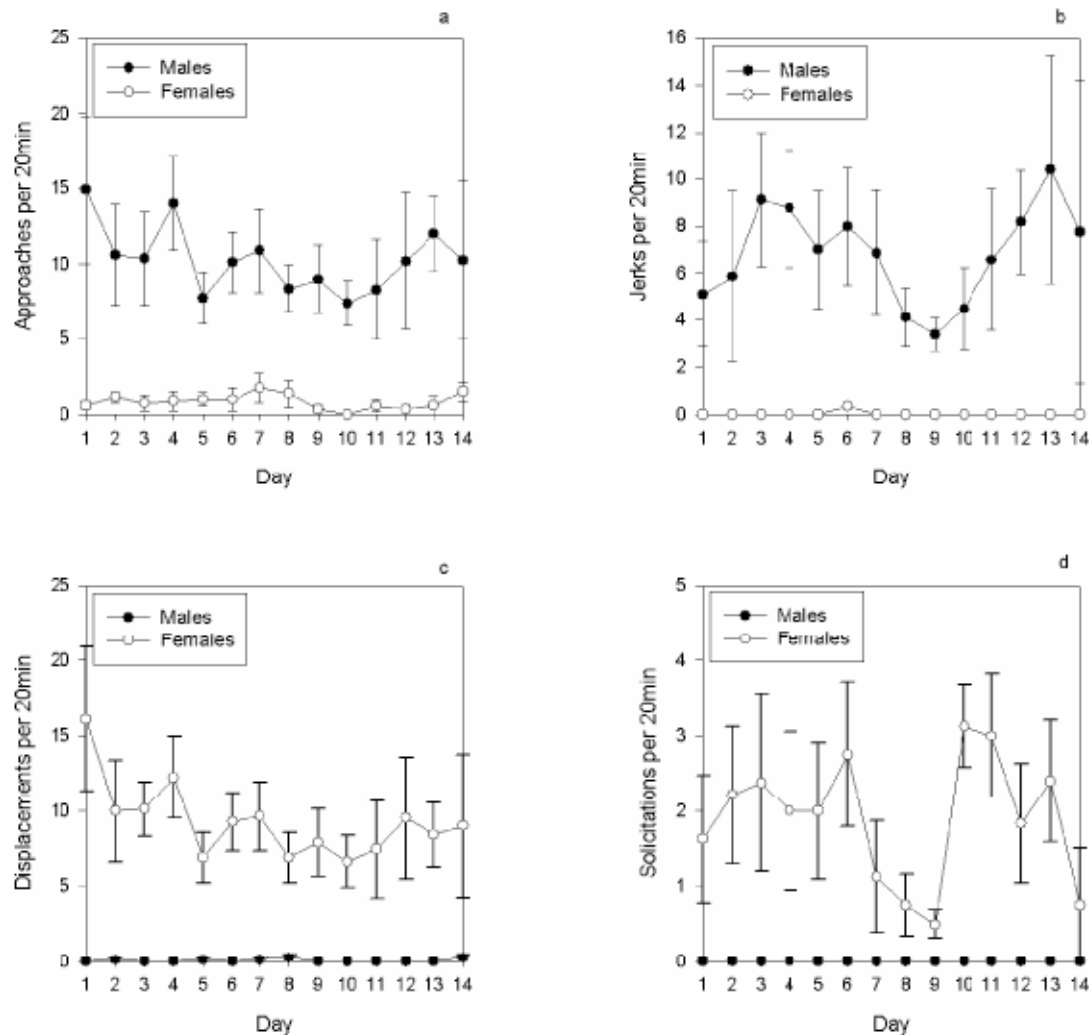
results suggest that female behavior has an impact on the rate of male behavior, something that would be very difficult to examine in a group with multiple females.

As in Reavis and Grober (1999), we used fertilized eggs as a marker for complete sex change and allowed the experiment to run for a maximum of 14 days. All tanks had eggs in the nest within the 14 days, but only half of those nests had eggs that remained until they could be verified as fertile. In the four other groups, the males did not display paternal care. In these instances, the male ate at least one clutch of eggs after day 10 of the experiment, thus making it impossible to use the presence of eyed eggs to verify fertility and thus terminate the experiment. The bulk of the data indicates that these eggs were viable. Clutch consuming males exhibited all male typical behavior (approaches, jerks, bites, and nips). Morphological examination revealed no observable differences between males that demonstrated appropriate care and those that did not. The gonads of the two groups were indistinguishable from one another, and both groups developed an AGS, the hallmark of the male sex. There were no differences in the papilla between the two groups, and all males displayed sexually dimorphic elongation of the longest dorsal rays. In addition, parenting males had fertile clutches at the same time as egg consumption was occurring, suggesting that the animals were capable of fertilizing eggs.

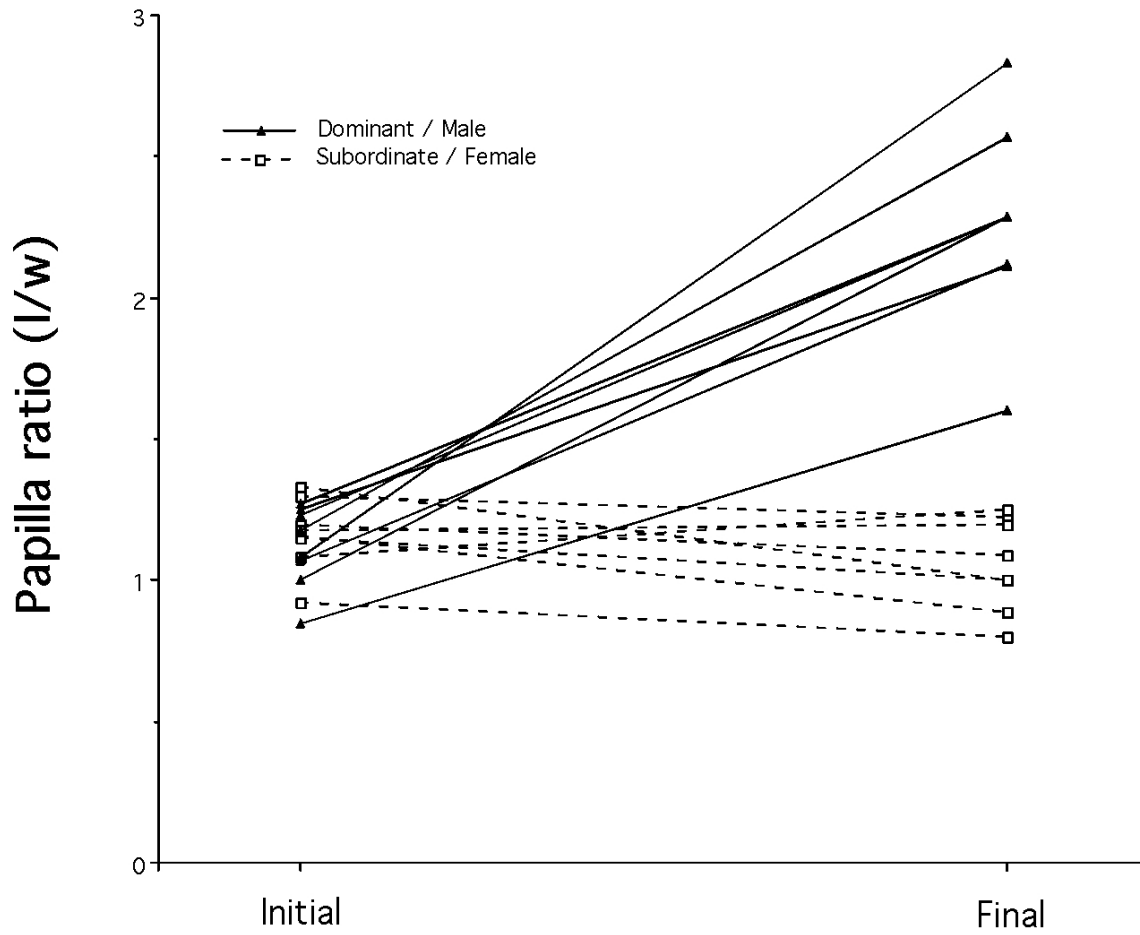
In experiments with groups of fish  $> 2$  (Reavis and Grober 1999), the amount of time required for full sex change was found depend on the size of the individuals in the group upon male removal. In groups where there was a greater than 10% size asymmetry between the top two females, sex change occurred more rapidly than groups where the size asymmetry was less than 10%. The observed delay was determined to be the result of an increase in time spent resolving the dominance relationship between the top two individuals. Individuals of similar size take longer to settle a conflict than when there is a size differential between contestants (Enquist et al., 1990; Koops and Grant, 1993). In this experiment we used pairs of size matched individuals; thus we were surprised to find that the contestants quickly resolved the dominance relationship. One possible explanation for this is that in a group, individuals may receive contradictory signals. This could result from

being dominated by one individual but also receiving positive stimulus by dominating other individuals in the group, thus prolonging conflict resolution. In dyadic contests, the signals are straightforward; one animal wins the encounters while the other loses them, thereby shortening the time and probably the number of interactions required for the formation of a stable dominance relationship.

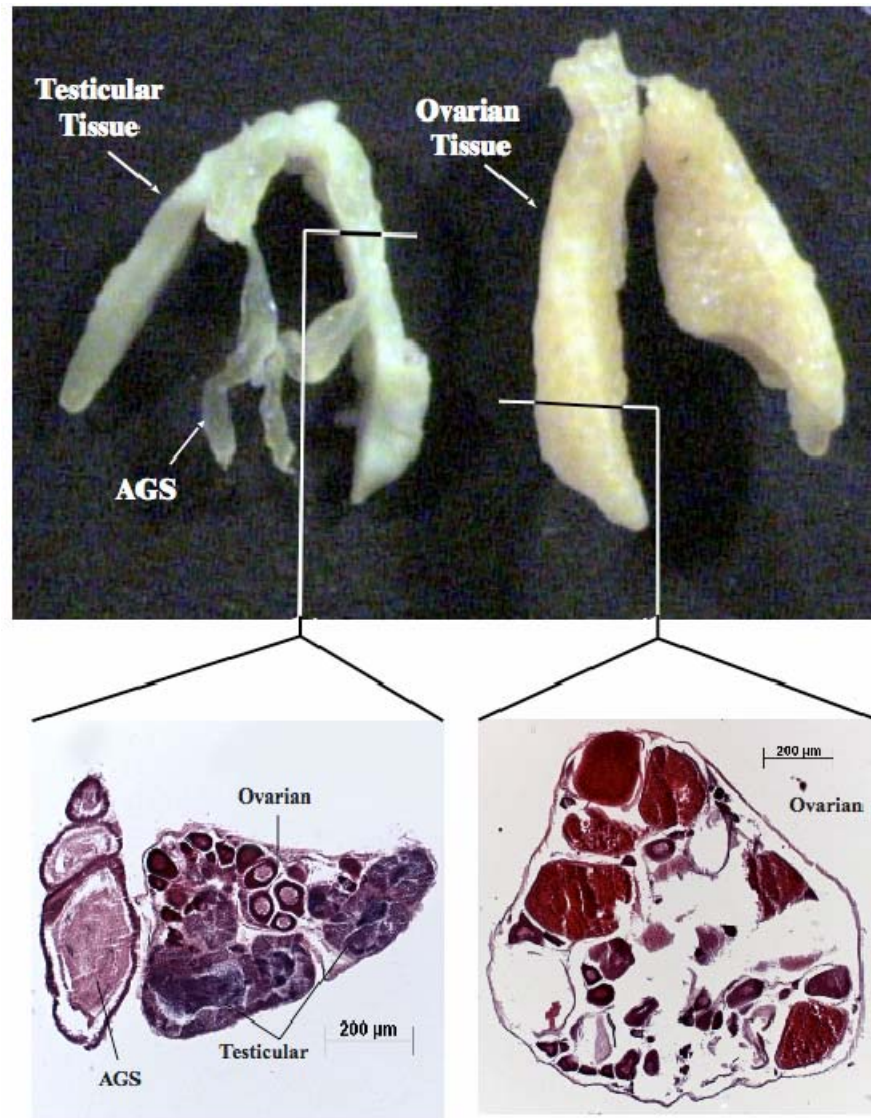
In summary, two females is a sufficient group size to induce sex change in *L. dalli*, as evidenced by one individual exhibiting male typical gonads, external genitalia, behavior patterns, and often fertilized eggs. When an *L. dalli* female becomes dominant, the first noticeable morphological change is the elongation of the dorsal fin. Dominant individuals also grew more rapidly.



**Figure 1.1:** Changes in sex typical behavior over the course of experiment. Animals that are labeled male are individuals that changed sex, i.e. those that would become male. a: Males approach and females rarely do, demonstrating dominance b: Jerking behavior, a major component of male courting behavior, is exclusively produced by males. c: females were displaced at high rates compared to males. d: solicitations, a type of female courting behavior, are exhibited exclusively by females. Note the similar pattern of behavior in figures b and d.



**Figure 1.2:** Papilla length/ width ratios at the start and conclusion for all animals in pairs of fish. Dominant/ males had a significantly higher ratio than subordinate/ females at the end of the experiment ( $t= 8.64$ ,  $df = 14$ ,  $p< 0.0001$ ).



**Figure 1.3:** Gonad morphology of a typical male (left) and female (right). The cross-section of each type is shown underneath. Note that the male gonad contains a majority of testicular tissue with some ovarian tissue in addition to an accessory gonadal structure (AGS). The female gonad contains only ovarian tissue and is visibly full of eggs.



## Chapter 2

Elevated 11-ketotestosterone during paternal behavior in the bluebanded goby (*Lythrypnus dalli*)

Rodgers, EW, Earley, RL, Grober, MS, (2006) *Hormones and Behavior*; 49:610-614

### **Abstract**

The relationship between androgens and paternal behavior is not straightforward, potentially because of the diversity of tasks a male must undertake to maximize reproductive success, notably alternating between courtship, aggression, and offspring care. In some species, these events are separated in time, but in others they are coincident. The endocrine profiles of species that simultaneously court, parent, and defend a nest, such as male bluebanded gobies (*Lythrypnus dalli*), are not well understood. We sampled a potent fish androgen, 11-ketotestosterone (KT), at different life history stages (experienced parenting males, experienced males not actively parenting, inexperienced males with their first clutch, and females), to examine this relationship. We found that experienced parenting *L. dalli* males have the highest KT levels of any group, while none of the other groups differed significantly. Males at two time points showed elevated KT levels when they have eggs compared to when they do not. Our data suggest that KT facilitates at least some aspects of parental care in *L. dalli*.

### Respective Contributions

Dr Earley aided with hormone assays and statistical analysis, while Dr Grober assisted with the analysis and editing of the following study and published manuscript.

## Introduction

In many species, androgens are elevated during some facets of male reproductive behavior, namely courtship and aggressive behavior, and are depressed during others, such as paternal care (Oliveira et al., 2002; Wingfield, 1984; Wingfield et al., 1997). Although an inverse relationship between androgens and paternal care is present in many species (Ketterson and Nolan, 1994; Van Roo, 2004; Wynne-Edwards, 2001), it is not evident in all paternal species (Ros et al., 2004; Trainor and Marler, 2001; Trainor and Marler, 2002; Ziegler et al., 2004; Ziegler et al., 2000).

One common component of paternal care that may complicate the androgen relationship is aggression (Marler et al., 2003). Males of many species must actively defend a nest or territory while providing parental care. This necessity for aggressive behavior may preclude a decline in androgen levels during paternal care. Fish provide a unique opportunity to test these ideas, because many species exhibit high levels of paternal care that co-occur with aggression.

11-Ketotestosterone (KT), a potent androgenic steroid in fishes, activates a suite of male typical behavioral and morphological traits in most fish species, including sex changing species and species exhibiting alternative male reproductive tactics (Oliveira, 2004). Interestingly, the territorial male phenotype in species exhibiting alternative reproductive tactics often provide exclusive care of offspring and as a rule have higher levels of KT than sneaker or satellite males (Brantley et al., 1993; Oliveira et al., 2001a). KT has been shown to be the androgen that mediates the challenge response in fishes (Hirschenhauser et al., 2004). The role of androgens in paternal care in fishes has generally been addressed in two ways: by sampling endogenous androgens over the course of the mating cycle, or administering exogenous steroid hormones to parenting males. These studies have yielded mixed results. Many studies of endogenous androgens document a decline when a male fish is parenting, most often following an initial period of high androgens (Knapp et al., 1999; Oliveira et al., 2002; Pall et al., 2005; Pall et al., 2002a; Pankhurst, 1990; Sikkel, 1993; Specker and Kishida,

2000), while exogenous administration of androgens does not appear to inhibit paternal care to a substantial degree (Kindler et al., 1991; Pall et al., 2002b; Ros et al., 2004) and can facilitate parenting in the blue gourami (Kramer, 1972).

An important question then is how androgens might mediate a trade-off between aggression and paternal care in species that simultaneously defend a territory/ nest and care for offspring. The sex changing fish *Lythrypnus dalli* is an excellent model system in which to examine how KT might impact behavior in different life history stages. Females do not provide any parental care and will consume eggs if allowed. Dominant *L. dalli* females change sex following male removal, and males exhibit parental care that often co-occurs with, but at times is dissociated from (e.g., when no clutches are present) nest defense. Males in this species are territorial, parental, and polygynous, mating with all females in their harem. Like many marine teleosts, *L. dalli* has a pelagic larval phase that begins immediately following hatching (Steele, 1997), and thus male parental care ends when eggs hatch. *L. dalli* also allows for comparisons of non-parenting females and inexperienced males to parenting males, and examination of whether KT levels are altered as a non-parenting female changes sex to a functional male that provides exclusive parental care.

We hypothesize that females will have the lowest KT levels, with inexperienced and experienced males have higher levels respectively. Because *L. dalli* males must continue to defend their nest aggressively and court additional females while parenting, we predict that KT will not decline when they are providing parental care.

## Materials and Methods

### *Subjects*

Twenty-six groups of 4-5 individuals were established and allowed to co-habitate and reproduce for at least 2 months. Groups consisted of 1 large male ( $38.1 \pm 0.5$  s.e.m.) and between 3 and 4 smaller females. Sex was determined by examination of the external genital papilla (Behrents, 1983). *L. dalli* females have a papilla length-to-width ratio of approximately 1.0 whereas males have a ratio of 1.6 or greater (St. Mary, 1994). Each group in the study was determined to be reproductively and parentally competent, as measured by successful rearing of eggs by the male to the eyed larvae stage. Groups were kept at 18.3°C, with 12h light: 12 h dark photoperiod, and fed frozen brine shrimp twice daily. Animals were housed in 38 L aquaria, each with an individual filter system (Marineland). Fish were collected (California Fish & Game permit # 803034-01) off the coast of Santa Catalina Island, California, using an anesthetic solution of quinaldine sulfate (Sigma Chemical) and hand-nets. The fish then were transported back to the laboratory at Georgia State University, Atlanta. This research was carried out in accordance with the IACUC standards for use of animals in research at Georgia State University.

### *Experimental Design*

Parentally experienced males were sampled for water borne KT at two time points: when they were parenting (24 h after the appearance of a new clutch), and when they were between clutches (24 h after hatching a clutch, when no eggs were present). Egg status (presence or absence) was recorded each day for all groups. Samples for water-borne KT were collected by removing the male and the nest tube from the home tank, and placing the male in 100 ml of freshly mixed seawater (DI water with Instant Ocean™, ~1.022 specific gravity) for 1 h. To prevent the remaining females from consuming eggs while male is being sampled, the nest tube was placed in a separate container filled with water from the home tank. At the conclusion of sampling, the male and the tube were returned to the tank. Sampling order was balanced, with half the males being sampled first

while parenting and half the males sampled first between clutches. There was no effect of sampling order on KT levels (paired t-test,  $t_{21} = 0.339$ ,  $P = 0.737$ ). In addition, there was no correlation between body size and KT ( $R^2 = 0.002$ ,  $F = 0.064$ ,  $P = 0.8$ ). Hormone sampling was conducted at the same time each day, between 1000 and 1100h. We noted that in several cases ( $n = 8$ ) eggs were consumed within 24 h after the parenting sample. Twenty-four of 26 males were sampled for both time points. The other two males never were without eggs in the 3 weeks of the study.

In the second part of the study, we collected hormone samples from females and new “parentally inexperienced” males. To do this, we removed the two largest females in 12 of the group tanks described above. We placed them in individual beakers for hormone collection as described above. When the samples were completed, we returned the females to their respective tanks and removed the existing male. This will induce the largest female to change sex into male. The same two fish (now the new male and female) were then sampled again at the first appearance of eggs, following the procedure described above.

### *Hormone Assays*

Steroids were extracted from 100 ml of water using Lichrolut C18 columns (Carlisle et al., 2000), and the hormone was eluted from the column with 4ml of methanol. The methanol was then evaporated in a vacuum centrifuge at 40°C and re-suspended in 110 µl of assay buffer (Greenwood et al., 2001). KT levels were assessed using commercially available KT EIA kits (Cayman Chemicals Inc.). All samples were run in duplicate, and all three 96-well assays were conducted on the same day. Intra- and interassay coefficients of variation were derived from two pooled samples of *L. dalli* water extract (see below) included in each assay. Intra-assay coefficients of variation were 2.13%, 1.74%, and 7.9%, and the interassay coefficient of variation was 7.2%. Samples were excluded from analysis if they exceeded the uppermost values on the serial dilution and standard curves.

The kit was validated for *L. dalli* by assessing parallelism of a serial dilution curve with the standard curve and quantitative recovery. Hormones were obtained and extracted from 48 non-experimental fish (males and females) using a method similar to that described above (collection period of 8 h). The evaporated samples then were re-suspended in 60 µl 0.1M phosphate buffer and combined into a pool of 2.9 ml. The pool was kept either at 1:1 (for serial dilutions) or diluted 1:16 in EIA buffer aliquoted and frozen; the aliquots were used for dilutions and quantitative recovery.

210 µl of the pooled, 'neat' (1:1) control was used for the serial dilutions. Briefly, 105 µl of this sample was transferred to a 1.5 ml microcentrifuge tube and mixed (by vortexing) with 105 µl of EIA buffer to create a 1:2 dilution; 105 µl of 1:2 dilution was mixed with an equal volume of EIA buffer to create a 1:4 dilution, and so on until 1:64. The serial dilutions were run in duplicate. The log-logit transformed dilution curve was constructed using average %B and pg/ml for the seven samples. The dilution curve was parallel to the standard curve (comparison of slopes:  $t_{11} = 0.001$ ,  $p = 0.99$ ; Zar 1996, p. 355).

A large (560 µl) sample of the goby pooled control was used for quantitative recovery. 100 µl of this large sample was pipetted into a tube to constitute the 'neat' control. 70 µl of the large sample was then pipetted into 8 additional tubes and mixed with an equal volume of one of the following standards (obtained from the Cayman Chemicals, Inc. KT EIA kit): 0.78, 1.57, 3.13, 6.25, 12.5, 25, 50, 100 pg/ml. Expected recovery concentrations were based on the known amount of KT in the *L. dalli* control sample (e.g., known *L. dalli* concentration + 25 pg/ml divided by 2). Minimum observed recovery was 92.6%. The slope of the observed vs. expected curve was 1.029, indicating a highly linear relationship between observed and expected recovery ( $F_{1,7} = 832.4$ ,  $p < 0.0001$ ,  $R^2 = 0.99$ ).

### *Statistics*

The data were normally distributed and thus were analyzed using parametric statistics. For independent samples, analysis of variance (ANOVA) was conducted and, when applicable, post hoc analyses were conducted using Tukey's HSD. Comparisons were made between females, inexperienced males, and either experienced parenting males or experienced non-parenting males. Paired t-tests (two tailed) were used to compare individuals sampled twice (experienced males: parenting vs. non-parenting, new males: as female vs. new clutch as male, and female sampled twice, when established male was removed then again when new male had first clutch). Linear regressions were also performed to assess the potential relationship between KT and body size. Logistic regression was performed to assess the influence of both KT concentration and experience on the probability of post-sampling egg consumption. Significance level in all cases was set at  $P < 0.05$ . Mean values  $\pm 1$  standard error are given. All analyses were carried out using JMP v5.0.1 (SAS Institute Inc.). KT concentrations are represented as pg/ sample (= pg/ ml \* ml of reconstitution buffer).

### **Results**

Experienced parenting males showed significantly higher KT concentrations ( $76.51 \pm 6.91$  pg/ sample) compared to inexperienced males ( $38.77 \pm 11.77$ ), and females ( $32.83 \pm 12.27$ ) (ANOVA,  $F_{2,46} = 6.41$ ,  $P = 0.0036$ , post hoc: Tukey's HSD,  $p < 0.05$ ). KT concentrations were not significantly different between experienced non-parenting males, inexperienced males, and females (ANOVA  $F_{2,44} = 2.138$ ,  $P = 0.13$ ). Paired comparisons from individuals sampled twice show that established males ( $n = 23$  pairs) had significantly elevated KT concentrations when parenting compared to when there were no eggs in the nest (Figure 2.1; paired t-test,  $t = 2.64$ ,  $P = 0.015$ ). Individuals who were sampled as females then again males with their first clutch

(n= 8), did not show significant differences, but the new males tended to be lower (paired t-test,  $t = 2.22$ ,  $P = 0.06$ ). Females sampled twice (n= 10), showed no differences in KT levels (paired t-test,  $t = 0.533$ ,  $P = 0.61$ ).

Egg consumption was noted after the “parenting” sampling in 8 of 26 experienced groups and 10 of 11 inexperienced groups. Using a logistic analysis we examined whether KT concentrations and/ or parental experience could predict the probability of egg consumption after sampling. There was not a significant relationship between parenting KT levels and egg consumption (L-R  $\chi^2 = 2.61$ ,  $P = 0.11$ ). The relationship between experience and egg consumption was significant, with experienced males being less likely to consume their clutch after sampling (L-R  $\chi^2 = 15.07$ ,  $P = 0.0001$ ).

## Discussion

Experienced parenting males had higher KT levels than females or inexperienced parenting males, or those same males when not parenting. We predicted that KT would not decline during parenting, potentially stemming from a male’s need to aggressively defend the nest, but we had little reason to predict that KT would increase during parenting in a stable group.

KT levels did not differ between females, inexperienced males (still possessing a large amount of female gonadal tissue), and experienced males (negligible or no female gonadal tissue) that were not parenting. Females who became males had showed no increase in KT, and there was a trend toward a decline in KT. This is somewhat surprising considering the dramatic masculinizing effects of KT on female morphology in this species (Carlisle et al., 2000). However, it has been found that males and females have similar hormone levels in *Gobiodon histrio* (Kroon et al., 2003). Another possible explanation is that KT acts to masculinize the physiology during sex change, but returns to baseline levels once the functional male phenotype has been achieved. While the physiological KT levels recorded in this study are far lower than levels produced by exogenous administration, the role of KT in the expression of sexual phenotype in this species remains unclear.



In *L. dalli*, KT is not incompatible with paternal behavior. In species where a high degree of male parental care is required for successful rearing of offspring, insensitivity to testosterone has been observed (Hunt et al., 1997; Lynn et al., 2002; Lynn et al., 2005; Van Duyse et al., 2000). Paternal care is required in *L. dalli* for successful hatching. However, the observed increase in KT while males are actively parenting suggests that KT is positively associated with paternal care in experienced males. While this does not conform to the classic trade-off model of androgens in parenting males, it is consistent with evidence from a number of studies involving fish (Kindler et al., 1991; Ros et al., 2004) and rodents (Trainor and Marler, 2001), where it has been demonstrated that androgens either do not interfere with, or potentially facilitate paternal care (Kramer, 1972; Trainor and Marler, 2002). In cotton-top tamarins, parental experience has differential effects on male testosterone responses to female pregnancy, with more experienced males showing greater elevation of testosterone than less experienced males (Ziegler et al., 2004). Recent work in the bluegill sunfish has shown that androgens do not interfere with paternal care, and that males in the best condition maintained high androgens throughout the parenting phase (Magee et al., 2006).

In many species territoriality and courting are temporally disassociated from parental care, whereas in *L. dalli* these behavioral suites occur in concert. A positive correlation between androgens and paternal care in species that must simultaneously defend a nest, court females, spawn, and care for offspring has been predicted by Marler et al. (2003), and our findings in *L. dalli* clearly support their prediction.

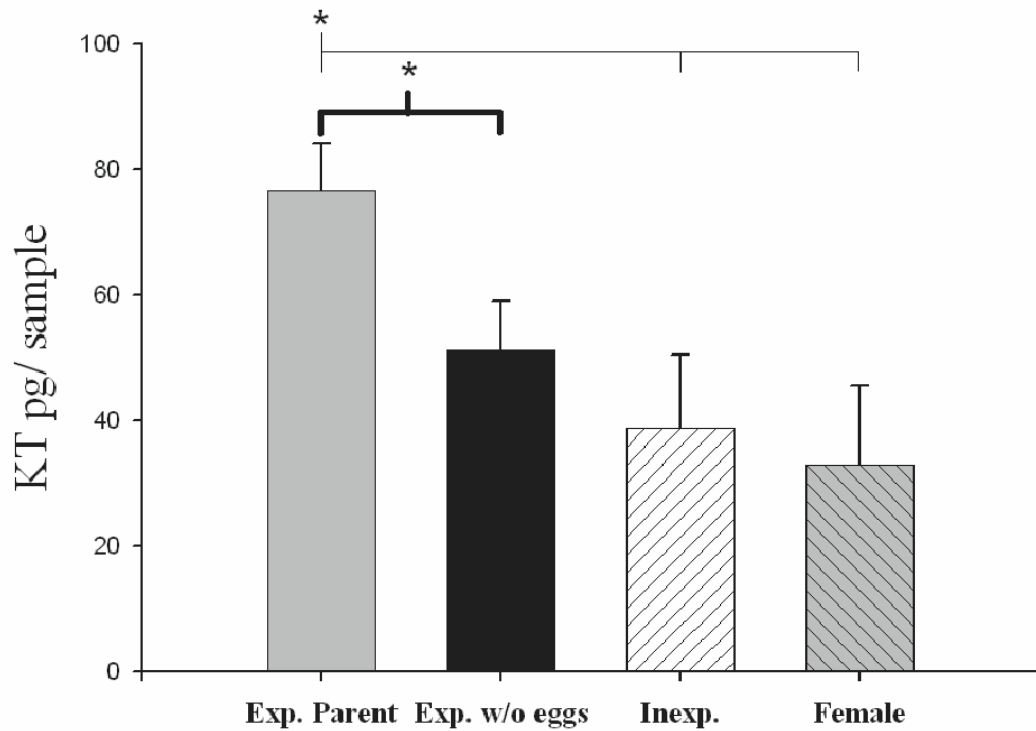
There are several possibilities for why KT may be elevated during parenting. The first is that KT is positively associated with elevated aggression (Ros et al., 2004) and males may need to raise their level of aggression to adequately defend the nest. While we did not examine rates of aggressive behavior in this study, it is known that *L. dalli* males exclude females, and potentially other males, from the nest regardless of presence of eggs (Black et al., 2005b; Rodgers et al., 2005). A male's need to constantly defend the nest should not interfere with his ability to parent appropriately in species that must do both simultaneously. *L. dalli* males

continue to court and spawn with females throughout any given brood cycle, and it may be this courting behavior that requires KT to remain high. In many fish species KT is high when males are courting and during the early stages of parental care (Knapp et al., 1999; Pall et al., 2002a; Sikkel, 1993). It is reasonable to assume that KT may contribute to high levels of courtship behavior or vice versa.

It is not known if KT levels change in *L. dalli* males across clutch development, such as in the plainfin midshipman (Knapp et al., 1999). In our laboratory, it takes approximately 5 days for the larvae to hatch, whereas it takes up to a month for midshipman eggs to reach the larval stage. Hormonal changes across the parental cycle have yet to be examined in *L. dalli*.

The probability of clutch consumption changes with experience but is not significantly correlated with KT levels. Experienced males tend to be less prone to clutch consumption following sampling disturbance than new males with their first clutch. The relationship, if any, between KT, experience, and clutch consumption is unclear. Experienced males have higher KT when parenting than inexperienced males and consume fewer eggs, but KT does not significantly change consumption probabilities. Other variables are likely to be involved in explaining this phenomenon.

In this study, we examined only levels of KT; we cannot predict the levels of other steroid hormones, such as estradiol or testosterone. Levels of other relevant hormones will be needed to paint a more complete picture of the role of steroid hormones in paternal care, but the present work provides interesting insights into the role of KT in paternal care among male fishes that must simultaneously defend a nest, court females, and parent. Androgen responses and their relationship to parental care have been shown to vary with mating system (Hirschenhauser et al., 2004; Wingfield et al., 1990) and parental necessity (Lynn et al., 2002; Lynn et al., 2005; Van Duyse et al., 2000). The hormone profiles in *L. dalli* likely reflect differences in life history in a polygynous species that must continue courtship and territorial defense while engaging in paternal care.



**Figure 2.1:** KT levels of different stages. Thin bars represent significant ANOVA comparisons; thicker bar represents significant paired t-test comparisons. Experienced parenting males (Parent) have significantly higher KT levels than experienced males without eggs (Non-parent) inexperienced males (Novice) and females. Experienced males without eggs have similar levels of KT to inexperienced males and females. When experienced males are parenting, they show significantly higher levels of KT than the same individuals when they do not have eggs (paired t-test). Post hoc conducted using Tukey's HSD. Error bars represent s.e.m.

### Chapter 3

Social status determines sexual phenotype in the bi-directional sex changing bluebanded goby (*Lythrypnus dalli*)

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#### **Abstract**

The goal of this study is to describe the behavioral mechanisms and patterns of protandrous sex change in bluebanded gobies (*Lythrypnus dalli*) and to compare it to the well-described behavior patterns of protogynous sex change. To do this, we established unisex groups of males and females, and recorded behavioral and anatomical changes over a 42 day period as they determined social status and sexual phenotype. Social status, rather than the expression of a particular behavior, accurately predicted final sexual phenotype. Rates of submissive behavior, but not aggressive behavior, were predictive of the discrete status classes. Multiple individuals changed sex simultaneously if their sexual phenotype and social status were discordant, a novel finding suggesting that once a social hierarchy is established, individuals determined their sexual phenotype, regardless of initial sex, based on a simple operational principle: if subordinate, express female; if dominant or not subordinate, express male. This work demonstrates that similar mechanisms underlie sex change in both directions in *L. dalli* and potentially other sex changing species.

#### Respective Contributions

Dr Earley aided in the observation, statistical analysis, and the editing of the following manuscript. Dr Grober contributed to the data analysis and editing of the manuscript.

## Introduction

Dominance status has far reaching consequences for social animals with regard to the reproductive success they can achieve (Ellis, 1995). The relationship between dominance and reproductive success provides a unique set of challenges for species in which the social environment regulates functional sex (Kuwamura and Nakashima, 1998). In the context of a social group, individuals must make critical life history decisions based on the accumulation and evaluation of local information. Questions regarding “why” sex change would arise in certain species are addressed, in part, by the size advantage hypothesis (Warner, 1988; Warner et al., 1975), which predicts the potential for, and direction of, adult sex reversal based on size-fecundity differences between the sexes. Because optimum “size at sex change” refers to the relative size of individuals in any given social group, and not absolute size (Warner, 1988), an individual must make a decision on when to change sex based on local information (Munoz and Warner, 2003). Although this information is inherently variable, because of potential immigration or emigration and life-long growth, these decisions are of great import, because fish will subsequently invest heavily in a new suite of sex specific traits.

Examination of the proximate mechanisms directing sexual allocation decisions will aid in developing a more complete understanding of the sex change process (Munday et al., 2006). One approach to better understand sex change is to identify the operating principles underlying allocation decisions, and in the case of bi-directional species, the degrees to which similar or different processes guide protogyny (female to male) and protandry (male to female). Agonistic interactions determine which individual will change sex in same-sex pairs of several bi-directional species (Kuwamura et al., 2002; Nakashima et al., 1995; Sunobe and Nakazono, 1993), and also direct protogynous sex change in *Lythrypnus dalli* (Gilbert), in both groups (Lorenzi et al., 2006) and pairs (Rodgers et al., 2005). Whether or not there is similar social regulation of protandrous sex change in *L. dalli* is not known. Tests of how dominance interactions regulate sexual transitions in both directions have been done only in pairs of fish (Kuwamura et al., 2002; Nakashima et al., 1995; Sunobe and

Nakazono, 1993), but many bi-directional species live in larger male dominated groups, which should pose a different set of challenges with regard to the reproductive payoffs associated with sex change. This study examines the behavior and morphology of all members of unisex social groups, both male and female, to assess the effects of behavior patterns, social status, and initial sex on the expression of sexual phenotype.

During protogynous sex change in all female groups, only the most dominant individual changes sex from female to male (Reavis and Grober, 1999). One possibility for protandrous change is that only the most subordinate male in unisex male groups becomes female, as has been seen in *Pseudolabrus sieboldi* (Ohta et al., 2003) and is consistent with data from St. Mary (1994) and Reavis and Grober (1999) in *L. dalli*. An alternative is that each individual responds to its own social status in a binary way, such that all individuals not achieving alpha status should change to female after social order is achieved. Through behavioral observations of unisex groups of males and unisex groups of females, we can evaluate whether the same cues regulate sex change in both directions.

## **Materials and Methods**

### *Experimental design*

In stable social groups, protogynous sex change in *L. dalli* can occur rapidly, with functional reversal (marked by presence of fertilized eggs in all female groups) taking approximately 2 weeks (Reavis and Grober, 1999; Rodgers et al., 2005). The time to sex change varies in groups according to the size symmetry between top ranking females; the closer in size the high ranking individuals are, the longer it takes for an individual to achieve alpha status and to change functional sex (Reavis and Grober, 1999). Thus, hierarchy establishment influences the time required and propensity to change sex. In the bluebanded goby, it has been shown that social dominance rather than size is an accurate predictor of which individual will change sex in the protogynous direction, when size asymmetries are minimized (Rodgers et al., 2005). When size asymmetries

are large, both size and social dominance are accurate predictors of protogynous change sex. To more closely examine the role of behavior and social dominance in protandrous sex change in this species, we ensured that one significantly larger individual was incorporated into each group to facilitate the emergence of a dominant individual in each test group.

### *Subjects*

Six unisex female groups composed of 4 individuals and six unisex male groups with 4 individuals were established. Prior to being placed into experimental groups, all animals were housed in small social groups of between 4-6 individuals per group with only one male. Individuals used in the all male groups were known to have spawned successfully with their group prior to inclusion in this experiment. Groups were housed in 38 L saltwater ( $\sim 1.022$  specific gravity) aquariums equipped with a gravel substrate, cylindrical nest tube (6 cm PVC pipe), biological filtration, and fluorescent overhead lighting with a photoperiod of 12 h light:12 h dark. Each group was completely (chemically and visually) isolated from the other groups. Fish were collected (California Fish & Game permit # 803034-01) off the coast of Santa Catalina Island, California, using an anesthetic solution of quinaldine sulfate (Sigma Chemical) and hand-nets. The fish then were transported back to the laboratory at Georgia State University, Atlanta. This research was carried out in accordance with the IACUC standards for use of animals in research at Georgia State University.

### *Group establishment*

All groups of *L. dalli* had one individual that was significantly larger (standard length) than the others (all-female:  $F_{1,21} = 29.6$ ,  $P < 0.0001$ ; all-male:  $F_{1,21} = 82.5$ ,  $P < 0.0001$ ) to facilitate one individual achieving dominance over the group. Standard lengths (mm) of the next two largest individuals in all-female groups were statistically indistinguishable (mean  $\pm$  s.e.m.:  $30.2 \pm 0.36$ , and  $28.6 \pm 0.14$ ; Tukey's HSD) but were significantly greater than the smallest animal of the group (mean  $\pm$  s.e.m.:  $26.8 \pm 0.95$ ; Tukey's HSD). In all-male groups, the standard lengths of the two smallest individuals were statistically indistinguishable (mean  $\pm$  s.e.m.:  $32.8 \pm$

0.33, and  $32.0 \pm 0.31$ ; Tukey's HSD) but were significantly smaller than the second largest animal of the group (mean  $\pm$  s.e.m.:  $34.9 \pm 0.66$ ). Variation in size among individuals was similar across groups (Levene HOV; all-female:  $F_{5, 17} = 0.86$ ,  $P = 0.53$ ; all-male:  $F_{5, 17} = 0.72$ ,  $P = 0.62$ ).

#### *Behavioral observation and status determination*

Agonistic behavior of each member of the all-female and all-male groups was observed during a 10 min focal period on eleven occasions throughout the experiment, and was recorded manually by one observer. Aggressive behavior represented a variety of behaviors: three forms of threat display (head-on and lateral display with fins and opercula flared, and 'headstand' display characterized by lifting of the tail off the substrate and wagging towards a nearby individual), and approach (orientation or slow movement towards another group member), as well as rapid approaches (attack) and bites towards another individual. Submissive behavior included avoidance (move  $< 2$  body lengths) or retreat (move  $> 2$  body lengths) from an aggressive act by another group member. Courtship consisted of rapid, zig-zag movements ("jerks") either towards another group member or around the nest. For each status class in the all-female and all-male groups, the proportion of total behavioral acts dedicated by each individual to aggression, submission, and courtship was calculated and used for statistical analyses to control for differences in overall performance of behavior across groups. Social rank was determined by directional aggression on each day of observation. In a given observation period, an animal was defined as dominant over another if it exhibited aggression towards, and elicited submissive behavior from the other animal without receiving aggression in turn. The sum of these individual interactions was used to determine the status of each animal in the group.

#### *Determination of sexual phenotype*

The genital papilla, an indicator of functional sex, was digitally imaged for each individual before the groups were established and every 2 weeks thereafter. The presence of fertilized eggs (visible appearance of eyes on the developing embryo) was used to verify that functional sex change had occurred within unisex



groups (Reavis and Grober, 1999). For papilla measurements during the experiment, all individuals were removed simultaneously from the aquarium, their papillae photographed, and then returned simultaneously to the aquarium; these brief disturbances did not disrupt the social hierarchy. Because the papilla is not perfectly reflective of gonadal state (St. Mary, 1994), assessment of sex by gonadal analysis was conducted at the conclusion of the experiment. Females possess a blunt papilla with a length to width ratio of approximately 1.0, whereas the male papilla typically has a longer, more pointed morphology with a ratio greater than 1.6 (Rodgers et al., 2005; St. Mary, 1994). All individuals of both sexes used in the experiment were within the normal papilla range for this species.

### *Statistical methods*

Mixed model repeated measures analyses of variance were conducted to examine changes in papilla morphology (length: width ratio) and behavior over time. One-way analyses of variance were used to determine behavioral differences among the status classes. Because the various behavioral scores for each individual are not independent, multiple one-way analyses were conducted in lieu of a multivariate analysis. Thus the P-values derived from the one-way analyses were subjected to sequential Dunn-Sidak adjustments to control for compounding Type I error (adjusted  $\alpha$ -values are reported in Results). All-female and all-male groups were treated separately because there was no significant status x group-type interaction (all behavior:  $F_{3,38} < 2.19$ ,  $P > 0.10$ ). Tukey's honest significant difference (HSD) was used as a post-hoc multiple comparisons test to determine status-dependent behavioral repertoires. All behavioral and morphological data were distributed normally without transformation (Shapiro-Wilk:  $W > 0.92$ ,  $P > 0.07$ ). Analyses were conducted using the JMP 5.0.1 statistical package and SAS version 8.2 (SAS Institute, Inc. 2002; Cary, NC).

## Results

Male-typical courtship behavior (jerks) was performed entirely by the alpha individual regardless of initial sex after hierarchy formation (Figure 3.1a & b). On the first day of the experiment, an individual who would eventually become beta, but whose status was unclear at the time, displayed jerking behavior, which was not observed in any other observational period. The four status classes could not be distinguished on the basis of total aggression in all-female groups (ANOVA,  $F_{3,19} = 13.04$ ,  $p < 0.0001$  ( $\alpha_{adj} = 0.017$ ), Tukey's HSD:  $\beta = \alpha$ ;  $\alpha = \gamma$ ,  $\alpha > \delta$ ;  $\beta > \gamma = \delta$ ; Fig 1a), or all-male groups (ANOVA  $F_{3,19} = 29.56$ ,  $p < 0.0001$  ( $\alpha_{adj} = 0.01$ ), Tukey's HSD  $\beta = \alpha > \gamma > \delta$ ; Figure 1.1b). Submission uniquely defined the four status classes in both all-female (ANOVA  $F_{3,19} = 34.98$ ,  $p < 0.0001$  ( $\alpha_{adj} = 0.01$ ); Tukey's HSD:  $\delta > \gamma > \beta > \alpha$ ; Figure 3.1a) and all-male groups (ANOVA  $F_{3,19} = 86.61$ ,  $p < 0.0001$  ( $\alpha_{adj} = 0.01$ ); Tukey's HSD:  $\delta > \gamma > \beta > \alpha$ ; Figure 3.1b). Status-dependent patterns of submission over time were used to ascertain hierarchy stability. There was no significant overall effect of time (Repeated measures ANOVA  $F_{10,209} = 1.01$ ,  $P = 0.43$ ), and the relationship among the four status classes with respect to submission did not change with time (Repeated measures ANOVA, time x status:  $F_{10,209} = 0.86$ ,  $P = 0.57$ ) or with group type (Repeated measures ANOVA all-male vs. all-female; sex x time x status:  $F_{10,209} = 0.71$ ,  $P = 0.71$ ). Coupled with the fact that virtually no status reversals were observed, the analyses indicate that in the time frame of the experiment (42 d), *L. dalli* form stable linear hierarchies. None of the reversals that were observed involved individuals from the alpha status class.

Each group had only one dominant individual (alpha) emerge that remained or became male, indicated by no change in papilla ratio in male groups or a significant increase in papilla ratio in female groups, respectively (Figure 3.2a & b). The papillae of all members of the female groups (ANOVA,  $F_{3,22} = 0.86$ ,  $P = 0.48$ ) and male groups (ANOVA  $F_{3,20} = 0.83$ ,  $P = 0.49$ ) were not significantly different at the start of the experiment. For both male and female groups there was a significant status x time interaction on the change in the papilla ratio (Repeated measures ANOVA Females:  $F_{3,18} = 17.7$ ,  $P < 0.0001$ , Males:  $F_{3,11} = 2.86$ ,  $P = 0.0015$ ).

There were no significant differences between status classes at day 0 (Repeated measures ANOVA Females,  $F_{3,22} = 0.86$ ,  $P = 0.48$ ; Males:  $F_{3,20} = 0.83$ ,  $P = 0.49$ ), but at 14 days (Repeated measures ANOVA Females:  $F_{3,22} = 20.84$ ,  $P < 0.0001$ ; Males:  $F_{3,20} = 6.83$ ,  $P = 0.0032$ ), 28 days (Repeated measures ANOVA Females:  $F_{3,22} = 99.27$ ,  $P < 0.0001$ ; Males:  $F_{3,20} = 25.87$ ,  $P < 0.0001$ ), and 42 days (Repeated measures ANOVA Females:  $F_{3,21} = 120.1$ ,  $P < 0.0001$ ; Males:  $F_{3,14} = 10.96$ ,  $P = 0.0012$ ) there were significant differences between status classes. In both directions, beginning at day 14, alphas had a greater papilla ratio than all other classes but there were no significant differences among the subordinate status classes.

There was a marked similarity between unisex groups in the mean time to first appearance of fertile eggs (functional sex reversal);  $16.5 \pm 0.99$  (s.e.m.) days for female groups and  $17.2 \pm 0.92$  (s.e.m.) days for male groups. Five of 6 male groups produced fertile eggs. Alpha individuals from several of the male groups maintained eggs in their nests continuously for the remainder of the experiment, evidence of highly successful groups with multiple females contributing eggs. Subordinates (ranks beta through delta) in all groups displayed female-typical papillae on the day eggs first appeared in the nest (Figure 3.2). In the male groups, female-typical gonads were verified for 14 of 17 subordinates; 3 subordinates possessed ambiguous gonads (a mix of male and female tissue).

## Discussion

Our experimental design reliably produced stable social hierarchies. Status within the social hierarchy dictated the expression of sexual phenotype: one individual per group, the alpha individual, remained or became male, while all subordinate individuals in the group ( $\beta$ - $\delta$ ) remained or became female. Both sexes demonstrated equivalent potential to establish stable social groups and to alter their sexual phenotype accordingly under these conditions. The remarkable temporal symmetry for functional sex reversal

(approximately 17 days in both males and females) suggests that both sexes are equally sensitive and responsive to perturbations of their social environment.

In the unisex male and female groups, a linear hierarchy was formed, wherein individuals tended to aggress toward those individuals to whom they were dominant and submit to those to whom it was subordinate. Proportions of submissive behavior were highly correlated with the four discrete status classes (Figure 3.2), and thus are likely to be an organizing principle of these small social groups. Notably, proportions of aggressive behavior did not have the same predictive value. Courtship behavior appears to be predictive of alpha status class, but is confounded by the fact that all alphas are also males. As such, the expression of jerking (male courtship behavior), while a hallmark of the status class cannot be separated from status class itself in the context of this data set, and may not be required for the initial acquisition of alpha status. These distinct status classes gave rise to only two sexual phenotypes. Thus status information, as it relates to sexual phenotype, is dichotomized. A simple organizing principle for the determination of sexual phenotype emerges: if subordinate express female; if dominant or not subordinate, express male. While it has been shown in several species that sex change is under social regulation (Fricke and Fricke, 1977; Robertson, 1972), we have identified a simple yet powerful operating principle in this species that determines the sexual phenotype of each group member and we have demonstrated that all group members respond in accordance with the principle regardless of initial sexual state or social status. *L. dalli* uses a binary representation of social status to direct sex typical allocation of reproductive resources and behavior, in both directions.

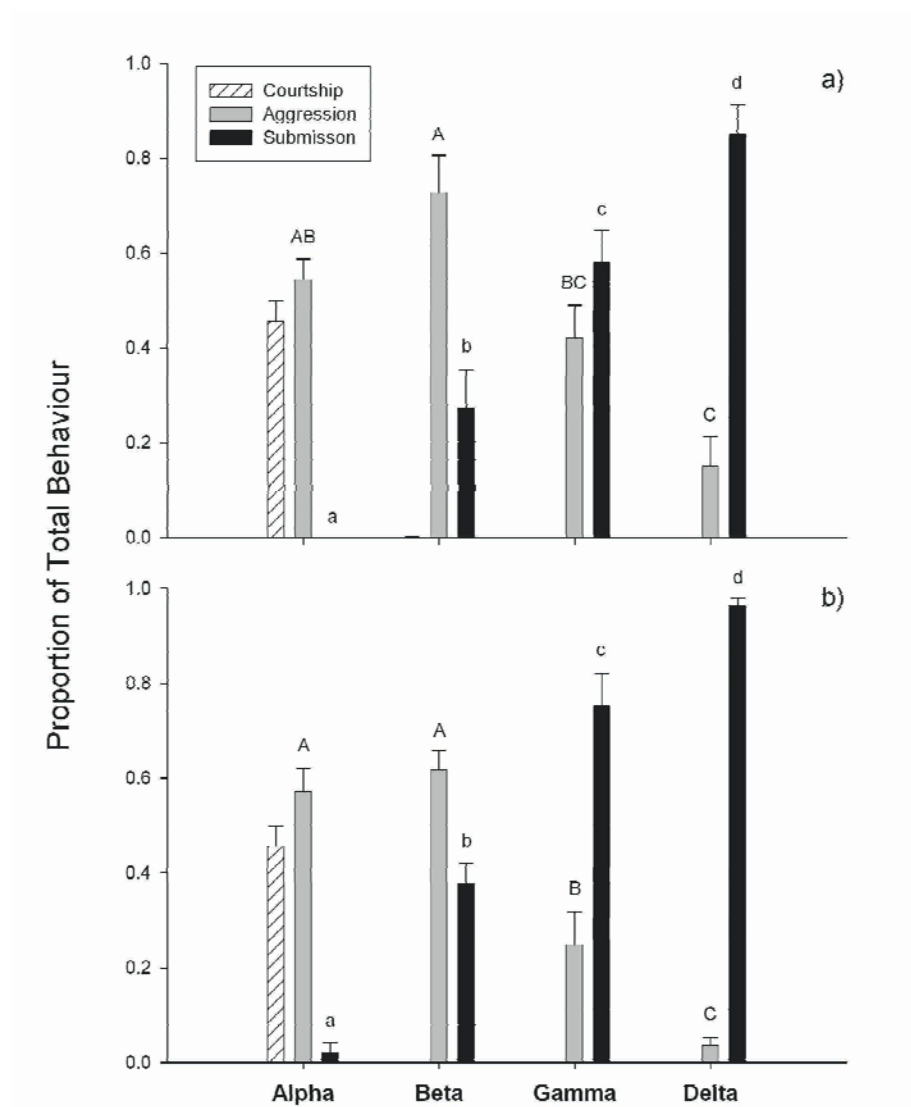
The results from this study contrast with earlier results in *L. dalli*, which indicated that sex change occurs more readily in the protogynous than in the protandrous direction (Reavis and Grober, 1999; St. Mary, 1994). The discrepancy between previous research on protandrous sex change in *L. dalli* and the present study may stem from differences in group composition, with our design facilitating the formation of a robust social hierarchy, which may be key to rapid changes in sexual phenotype. In social groups that are less tightly

ordered, ambiguity may result, which could explain instances of facultative sex change (Lutnesky, 1994; Ross, 1990).

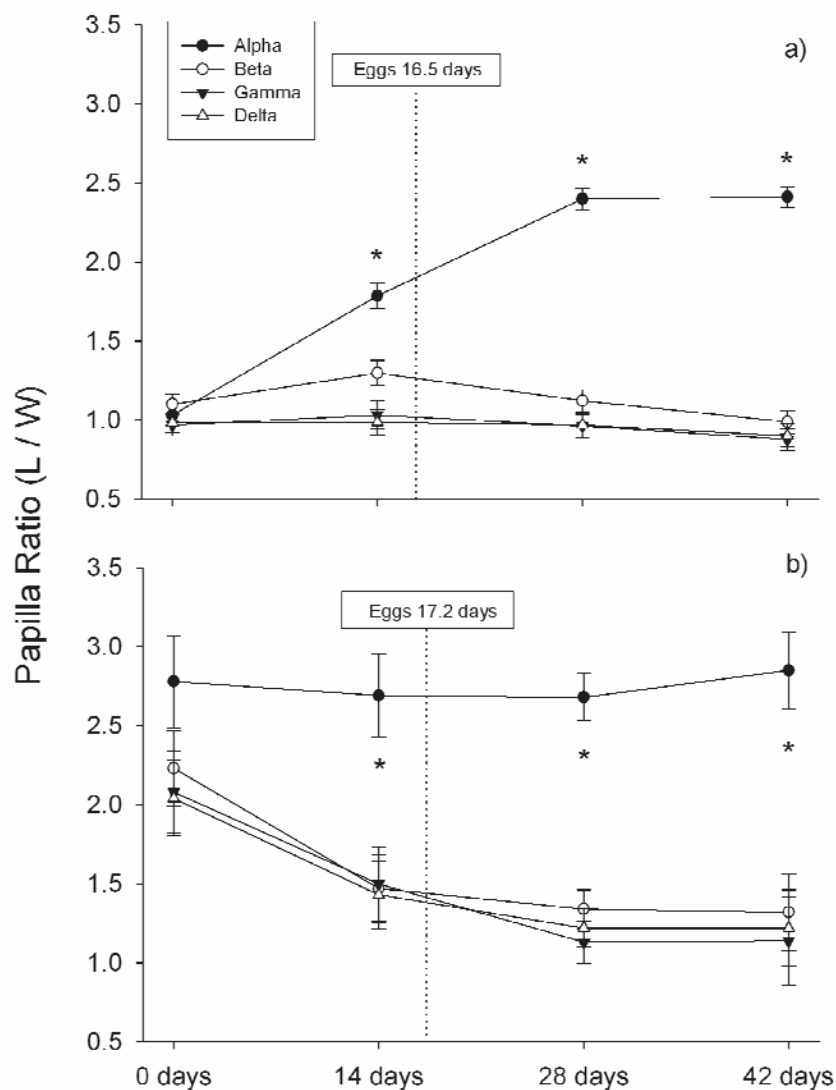
The striking parallelism between sex change in the protogynous and protandrous directions with respect to both the time course and the use of social status suggest that a similar mechanism may underlie both processes. Throughout evolutionary history, control over sexual differentiation pathways has been entrained to a variety of different signals (Zarkower, 2001). Social status provides an ideal signal in that regard, because of a pre-existing relationship between dominance and reproductive success (Ellis, 1995), and because young animals are unlikely to be dominant or to reproduce effectively as the resource holding sex. Such a mechanism could allow individuals to take advantage of the differences in size-fecundity skew between the sexes originally described in the size advantage model (Ghiselin, 1969; Warner, 1988; Warner et al., 1975). This suggests that sexual plasticity has been achieved over evolutionary history by shifting control of sexual differentiation from a static persistent genetic signal to a signal that may vary over the life of an individual, the variable signal model. Thus both unidirectional and bi-directional sex-changing fishes are likely to use the conserved process of sexual differentiation that is now linked to a variable signal (in this case, social status) to direct adult re-allocation of the suite of traits that characterize the sexes. Entraining sexual differentiation pathways to signals that are predictive of changes in reproductive opportunity may explain the persistence of sex change across a range of species and mating systems that are not subject to dramatic size-fecundity skew (Kuwamura et al., 1994) but where phenotypic flexibility would be reproductively advantageous, such as after mate loss or social disruption (Munday et al., 1998; Nakashima et al., 1995; Sunobe and Nakazono, 1993).

The operating principle described in *L. dalli* (if subordinate, express female, if dominant or not subordinate, express male) is unlikely to apply to all sex changing fish (Fricke and Fricke, 1977; Shapiro, 1988). However, it may apply to a large percentage of bi-directional species (similar social regulation has been documented in pairs of *Trimma okinawae* (Sunobe and Nakazono, 1993), *Labroides dimidiatus* (Kuwamura et

al., 2002), and possibly *Gobiodon histrio* (Munday, 2002). The principle, with its emphasis on subordination, will provide a model to examine the bi-directional potential of other sexually plastic species. The variable signal model, however, which allows for the control of sexual differentiation pathways by novel inputs, is likely to be broadly applicable to sexually plastic fishes.



**Figure 3.1:** Proportion of total behavior spent in each of 3 types of behavior, aggression, submission, and courtship. Top panel (a) represents the behavior of groups that began as females, and the bottom panel (b) represents the behavior of the groups which began the experiment as males. Bars represent status class mean  $\pm$  s.e.m. Aggressive behavior alone does not uniquely define status class, whereas submission does (see results). Bars noted with different letters are significantly different at  $P < 0.05$  using Tukey's HSD (aggression: capitals; submission: lowercase)



**Figure 3.2:** Changes in papilla morphology (length: width ratio) over the course of the experiment in A) all-female, and B) all male groups. Females possess a blunt papilla with a length to width ratio of approximately 1.0, whereas the male papilla typically has a longer, more pointed morphology with a ratio greater than 1.8 (Rodgers et al., 2005; St. Mary 1994). The first appearance of fertilized eggs is marked with a dotted line. Asterisks indicate a significant difference (Tukey's HSD,  $P < 0.05$ ) in papilla ratio between alpha and all subordinates. Error bars represent standard error.



## Chapter 4

Hormone administration can alter sexual morphology but not behavioral phenotype in a sex changing goby

*(Lythrypnus dalli)*

### **Abstract**

Steroid hormones have been implicated in the regulation of early sexual differentiation in all vertebrates and of adult sex change in fishes. Social environment is also recognized as a powerful mediator of sexual differentiation in many fishes. In this study, social environment was pitted against hormonal environment to explore their respective effects on the behavior and physiology of a bi-directionally sex changing fish. Fish were implanted with KT, E2, MT, T, and control, and then placed into either a socially permissive or inhibitory environment. Several behavioral and physiological traits were then observed and quantified. Social environment determined expression of both social and sexual behavior, regardless of hormonal implant or gonadal state. KT facilitates male reproductive physiology, but not behavior, while estrogen inhibits gonadal sex change but not behavioral sex change. Sexual phenotype is a constellation of correlated traits that respond independently to different influences. This calls into question the utility of using a single axis, either behavioral or physiological, to determine the sex of an individual in sexually plastic species, and suggests that future studies on the mechanisms whereby these independent traits are coordinated will provide key insights into the evolution and development of sexually plastic phenotypes.

## Introduction

Sex determination occurs early in development for most animals, at which time it is fixed for the remainder of the animal's life. This is not the case for all animals however, with many species including mollusks, crustaceans, echinoderms, polychaete worms and teleost fishes maintaining various degrees of sexual flexibility, into adulthood (Allsop and West, 2003; Charnov, 1979). Sexual plasticity, under appropriate conditions, can lead to dramatic increases in reproductive success (Ghiselin, 1969; Warner et al., 1975). A large group of advanced fishes, the teleosts, exhibit a stunning variety of often flexible approaches to sex that range from variable expression of sex specific behavior within sexual morphs, to perhaps most strikingly, rapid, reversible and fully functional adult sex change (Devlin and Nagahama, 2002; Kuwamura and Nakashima, 1998). Flexible strategies require the integration of external cues, in order to "choose" the appropriate sexual phenotype for a given set of external conditions, and a set of internal mechanisms to manifest that change in decision. Functional adult sex change again represents the most dramatic extension of this quandary, as incorrect choice can be so costly and the physiological change in phenotype so dramatic.

The integration of external cues in most cases involves assessment of local conditions, to varying degrees, the outcome of which determines whether an animal will retain its current sex or change to another. While there appears to be a generalized trend across all sex changing species that the optimal time to change to the resource holding sex is about 72% of adult body size (Allsop and West, 2003), at the level of the individual the decisions are more complex (Buston et al., 2004; Warner, 1988). Individuals must assess local cues that vary from species to species, and habitat to habitat. While it has been known for some time that sex in many species is under social control (Fricke and Fricke, 1977; Robertson, 1972), size (Warner et al., 1975), and in particular, relative size (Warner, 1988), has long been seen as the critical local information that determines sexual allocation. This has been challenged, on the proximate level, by studies that point to agonistic behavior (Kuwamura et al., 2002; Nakashima et al., 1995; Sunobe and Nakazono, 1993) or its extension, social status

(Rodgers et al., 2005; Rodgers et al., 2007), as the crucial information stream used for individual decisions. These ideas are not necessarily mutually exclusive, as increasing size asymmetries are very predictive of success in agonistic encounters. From a mechanistic perspective, however, the integration of behavior and sexual phenotype is likely to be more instructive. Using dominance or social status is an ideal cue, as it is correlated with reproductive opportunity and reproductive success across a very wide range of species and taxa (Ellis, 1995).

Changes in reproductive behavior and the assumption of opposite sex behavior is not dependent on gonadal hormones (Godwin et al., 1996). But these decisions do direct physiological transformations that involves changes in sex specific steroid hormones (Frisch, 2004). The present study focuses exclusively on protogynous sex change. Three major steroid hormones appear, in many species, to be involved in physiological, and possibly behavioral, transformation from the female to male sex: estrogens (E), testosterone (T), and 11-ketotestosterone (KT). A decline in estrogen at the onset is theorized to be a critical step in protogynous sex change (Nakamura et al., 2003). Estrogens are high during the female phase and decline rapidly over sex change in wrasses (Bhandari et al., 2003; Nakamura et al., 1989) and goby (Kroon and Liley, 2000). Treatment with aromatase inhibitors, which decreases circulating estrogen, induces partial or complete protogynous sex change in the honeycomb grouper (Bhandari et al., 2005), the blackeye goby (Kroon and Liley, 2000), and the threespot wrasse (Higa et al., 2003). Suggestive of its role as an inhibitory step in the process, when E2 is administered with either KT or an aromatase inhibitor, it prevents protogynous sex change (Bhandari et al., 2005; Higa et al., 2003). Aromatase also declines in the gonad during sex change in the Saddleback wrasse (Morrey et al., 1998). In addition to the relationship of gonadal aromatase to sex change, another potential source of estrogen regulation is the brain. Changes in brain aromatase are associated with sex change (Black et al., 2005a; Marsh et al., 2006), possibly through direct effects on circulating E2 levels, or alternatively through the facilitation behavioral changes (Balthazart et al., 2006).

The potent fish androgen KT (Borg, 1994) has been shown in several studies to increase over protogynous sex change (Bhandari et al., 2003; Cardwell and Liley, 1991; Nakamura et al., 1989) or is sexually dimorphic (Nakamura et al., 1989). Exogenous KT has been shown to masculinize many traits in sexually plastic species, including color (Cardwell and Liley, 1991; Semsar and Godwin, 2003) and external genitalia (Carlisle et al., 2000), to complete sex change (Higa et al., 2003; Kroon and Liley, 2000). KT has been shown to increase aggressive/ territorial behavior (Oliveira et al., 2001b) as well as induce male sexual behavior in female goldfish (Stacey and Kobayashi, 1996). KT is a potent masculinizing agent, but in several species of sexually plastic fishes it is either not different between males and females (Kroon et al., 2003; Rodgers et al., 2006) or low during sexual transitions (Johnson et al., 1998; Nakamura et al., 1989).

The role of testosterone, if any, in the sex change process varies by species and experimental manipulation. That is perhaps not surprising considering that both sexes of most species have detectable T (Frisch, 2004). In the saddleback wrasse, T does not vary over the course of sexual transition (Nakamura et al., 1989). Exogenous administration of T or a non-aromatizable form (17-alpha-methyltestosterone (MT)) induces sex change in some species (Yeh et al., 2003), but not in others (Kramer et al., 1988; Kroon and Liley, 2000). T has been shown to have opposite effects of promoting feminization and masculinization depending on the dosage (Yeh et al., 2003). The varied and contradictory effects of T administration have led to the theory that the primary mode of action for T is via its metabolites, E2 through aromatase and KT through 11-beta-hydroxylase and 11-beta hydroxysteroid dehydrogenase (Kroon and Liley, 2000).

In species where sex is determined via social interactions, two components are usually required (with particular focus on protogyny): release from inhibition and stimulation from other reproductive individuals (Robertson, 1972; Ross et al., 1983; Shapiro, 1979). Thus, these species tend not to change sex in isolation (Carlisle et al., 2000). In group living protogynous fishes, the general pattern of sex change is as follows; when

the dominant male vacates the group, the remaining females determine a new dominant individual, through agonistic interactions, who then transitions to become the new male (Robertson, 1972).

The Bluebanded goby, *Lythrypnus dalli*, is a group living bi-directional sex changer, with well-characterized behavior patterns (Black et al., 2005b; Reavis and Grober, 1999; Rodgers et al., 2005; Rodgers et al., 2007). In this species, social status established through agonistic interactions, determines sex in both directions, with dominant individuals being male and subordinates being female (Rodgers et al., 2007). Chemically manipulating sexually plastic individuals in a behaviorally permissive or inhibitory condition has been used to examine the relative influences of social environment and internal environment (Larson et al., 2003). To examine the relationship between social and hormonal cues associated with protogynous sex change in *L. dalli*, we placed individual females under varying social and hormonal conditions. The goal of this study was to examine baseline and exogenously elevated levels of sex steroids, and to determine the relationship between steroid hormones, behavior, and physiology. In the permissive social condition a female was placed with a smaller female, while for the inhibitory condition a female was placed with a larger female. Estradiol was administered to fish in a permissive condition, to test whether estrogen is inhibitory of physiological sex change in this species, and if behavior would also be inhibited/ feminized under permissive conditions. KT was administered under inhibitory conditions, to examine whether it would induce sex change under social circumstances that would not normally produce sex change and if KT may be sufficient to masculinize behavior, either through increases in aggressive behavior or the expression of male courtship behavior. T and MT were given under permissive and inhibitory conditions. These treatments will allow for assessing the direct role of T or whether its actions are mediated by its metabolites. Control, either sham or cholesterol, treatments will determine baseline sex steroid levels in the bluebanded goby. Dorsal fin length is known to be sexually dimorphic (Wiley, 1976) and fins grow more rapidly in fish undergoing protogynous sex change (Rodgers et al.,

2005), but it is unknown if this change is steroid dependent. Brain aromatase was measured to determine whether exogenous steroids alter the conversion of T to E2 in the brain.

## **Materials and Methods**

### *Subjects*

Initial and final sex of all individuals was determined by examination of the external genital papilla (Behrems, 1983). *L. dalli* females have a papilla length to width ratio of approximately 1.0 whereas males generally have a ratio of 1.6 or greater (St. Mary, 1994). Each group in the study was determined to be reproductively competent, as measured by successful rearing of eggs to eyed larvae. Groups were kept at 65°F, with 12h light/dark cycle, and fed frozen brine shrimp twice daily. Animals were housed in 33 L aquaria, each with an individual filter system (Marineland). Fish were collected (CF & G permit # 803034-01) on Santa Catalina Island, California, using an anesthetic solution of quinaldine sulfate (Sigma Chemical) and hand-nets.

### *Pairing and Implantation*

After measuring the standard length and capturing papilla images of a surplus of female fish, pairs were established, with a minimum of 3mm standard length difference between the two females in the pair. Hormone treatment was randomly assigned to the pair, with between four and six pairs in each group. Only one individual per pair received pharmacological treatment with one of the following substances, KT, MT, E2, T (Sigma Chemicals), Cholesterol, or Sham. Implants were made using a Parr Pellet press, which compacts hormone powder into a dense pellet. The pellet is then broken into smaller pieces and implanted into the body cavity. The incision was then sealed with cyanoacrylate glue, and the fish were returned to holding beakers for a one hour recovery period.

### *Behavioral Observation*

Fish pairs were given several hours to acclimate before behavioral observations began. Direct behavioral observations (10 min) were collected for each pair, each day, alternating between mornings

(approximately 9AM-11AM) and afternoons (approximately 2PM-4PM), for 10 days, with tanks watched in random order. Fish feedings, which occurred twice daily, were always completed immediately following the end of the observational period. All data were recorded using Stopwatch++ (Center for Behavioral Neuroscience, Atlanta, GA). To reduce potential bias all observers were blind to the treatment groups. The observer recorded the number of approaches, displacements, jerks, bites, time in the tube, and tail-waggles (Rodgers et al., 2005). An approach is defined as a fish moving within 5 cm of the other fish. Movement away from the approaching fish is recorded as a displacement. Jerks are a male typical behavior used during courtship (Behrens, 1983), and involve a saltatory swimming motion with movement laterally as well. Because jerks can be directed at the female or around the nest but not directed at a fish, a jerk towards a female is scored as both an approach and a jerk. Bites are aggressive interactions in which one fish bites the other after an approach. Time in nest, is measured from the time a fish enters the nest tube until it is greater than 50% out of the tube, and is calculated by Stopwatch++. Tail waggles are displayed by both sexes (Rodgers, 2005), and consist of a fish remaining stationary while moving its tail back and forth.

#### *Hormone collection and Analysis*

Water-borne hormone samples were collected by removing the individuals from their aquaria and placing them in 100 ml of freshly mixed seawater (DI water with Instant Ocean™, ~1.022 specific gravity) for 1 h. Steroids were extracted from 100 ml of water using Lichrolut C18 columns (Carlisle et al., 2000) and the hormones were eluted from the column with 4ml of ethanol or methanol, which was then evaporated from the samples with a Savant AES 1010 speedvac. Each sample was dried with a heat lamp for 45 minutes, and for an additional 45 minutes with no heat. RIA followed resuspension of the hormones in 60ul 0.1M phosphate buffer. 1ml of radiolabeled hormone was added to each sample, which was incubated in a 37°C water bath for 45 minutes. The radiolabeled hormone was then decanted from the tubes, the samples were dried for 30 minutes, and were then placed in a gamma radiation counter to determine hormone levels contained in each sample.

### *Aromatase assay*

After the one hour hormone sampling procedure, all animals were euthanized via exposure to excess MS-222 and then their brains were rapidly removed and frozen on dry ice. These tissues were stored at -80 °C until assayed. Following brain removal, the remainder of the bodies were immersion fixed in 4% paraformaldehyde. Frozen brain 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/h/mg protein after correction of the counts for quenching, recovery, blank values and percentage of tritium in  $\beta$ -position in the substrate.



## *Histology*

Gonads were surgically removed from the preserved bodies and prepared for histology by dehydrating and embedding them in paraffin using the ThermoShandon Citadel 1000. Gonads were sectioned at 7  $\mu$ m, using a rotary microtome. These sections were stored in a 37°C incubator until staining with Hemotoxylin and Eosin (Davenport, 1960). Gonadal sections were then analyzed under a light microscope for percentage AGS, testis, and total tissue using AxioVision image analysis software from Zeiss, Inc. Ten sections spaced throughout the gonad were analyzed when possible, as deemed sufficient for properly determining the sex of *L. dalli* (Drilling and Grober, 2005).

## *Statistics*

Where possible we utilized parametric statistics, including analysis of variance (ANOVA) and post hoc Tukey's honest significant difference (HSD) where appropriate. The distributions of the frequencies of the various behaviors for dominants and subordinates did not meet the criteria for parametric statistics (e.g. normality, homogeneity of variance), thus we used non-parametric methods to examine group differences. All behavioral analyses were conducted with the Wilcoxon signed rank test. Values for mean and median were similar, indicating a symmetrical distribution. The relationship between morphological traits and hormone levels were examined using simple linear regressions. Significance level in all cases was set at  $p < 0.05$ . Mean values  $\pm$  standard errors are given. All analyses were carried out using the JMP v5.0.1 (SAS Institute Inc.) unless otherwise noted.

## **Results**

### *Hormones*

In general, hormone data were as expected, implanted groups had higher levels of the hormone they were implanted with, however T was somewhat different than E2 or KT (see below). KT varied with treatment

group ( $F_{7,41}=12.85$ ,  $p<0.0001$ ), post hoc analysis reveals KT implanted fish have higher KT than any other group. Large and small T implanted groups in combination with large MT implanted fish formed a second group with slightly elevated KT. Estradiol produced similar results ( $F_{7,40}=6.29$ ,  $p<0.0001$ ), with the E2 implanted group significantly higher than all others. Testosterone was highly variable across groups, with both T implant groups along with 11 KT having significantly higher T than all other groups ( $F_{7,41}=5.73$ ,  $p=0.0002$ ) with the exception of large MT animals.

Control females and males differed with respect to E2 levels. Females had more E2 than did males ( $t=2.46$ ,  $p=0.02$ ). KT and T were not different among controls of either sex.

### *Behavior*

The social relationship between the individuals determined the behavioral pattern they expressed. The larger animals achieved dominance and displayed male typical behaviors while subordinate animals displayed female typical behavior in all treatment groups and instances. Large animals produced significantly more approaches ( $z=6.25$ ,  $n=53$ ,  $p<0.0001$ ), bites ( $z=5.56$ ,  $n=53$ ,  $p<0.0001$ ) and male courtship behavior ( $z=5.56$ ,  $n=53$ ,  $p>0.0001$ ; Figure 4.1). Dominants also spent significantly more time in the nest tube ( $z=4.59$ ,  $n=53$ ,  $p<0.0001$ ). Small animals were displaced significantly more than large individuals ( $z=-5.87$ ,  $n=53$ ,  $p<0.0001$ ). Tail waggle behavior was not specific to either dominants or subordinates. Owing to the robust impact of social circumstance on behavioral expression, to explore the effects of hormone treatments on behavior, groups were split into dominants and subordinates and analyzed separately using analysis of variance. Only rates of displacement showed a significant treatment effect, and only among the subordinates ( $F_{3,21}=4.57$ ,  $p=0.015$ ). Post hoc analysis shows that small KT and MT implanted fish were displaced significantly less than controls, and that T implanted fish were not different than either of the other groups. No other behaviors were significantly impacted by behavioral hormone treatment. E2 did not inhibit behavioral sex change in a permissive environment and KT administration was insufficient to sex reverse female behavior (Figure 4.1).

## *Morphology*

Two factors known to affect morphology were juxtaposed within the context of this experimental design, those being social status and steroid hormones. As a result there is a high degree of variation across groups, when separated by treatment, with only one dramatic result, that KT masculinizes the tissue of female fish. Four measures of “maleness” were used to determine the effects of steroids vs. behavior on morphology, dorsal fin length, papilla, percent testis, and percent AGS.

Exogenous hormone treatment did have a significant effect on reproductive morphology. E2 administration inhibited morphological female to male sex change (Figure 4.2), demonstrable through its feminization of the papilla (mean =  $1.23 \pm .177$  s.e.m.), the gonad, and the complete lack of AGS formation. The lack of an AGS precluded more detailed analysis of that trait. KT correlated with degree of masculinization of the gonad ( $R^2 = .27$ ,  $p = 0.01$ ) and papilla ( $R^2 = .13$ ,  $p = 0.0094$ ). This relationship disappears when examining only the non-manipulated animals. Control animals showed no correlations between circulating hormone levels and phenotypic traits (Figure 4.2). There is no relationship among controls between KT levels and papilla, male gonad, and AGS. Treatment with T or MT had minor masculinizing effects

Hormone treatment did not affect fin length in either direction in this experiment, nor did any individual hormone measure correlate with change in fin length. Fin length was significantly affected by social status ( $t = 3.53$ ,  $p = 0.0007$ ), independent of hormone levels, with dominant individuals having significantly greater elongation of the dorsal fin. Change in dorsal fin length was used as the primary measure because there was a significant difference at the start of the experiment with respect to fin length between large and small fish. Hormone treatment did not affect growth rate (ANOVA,  $p = 0.66$ ), but interestingly, neither did social status when compared across all treatments ( $t = .39$ ,  $p = 0.7$ ). Control individuals and their pairs did show significant growth differences between status classes ( $t = 2.28$ ,  $p = 0.035$ ), with dominant animals exhibiting more growth.

### *Aromatase activity*

Brain aromatase activity was not significantly different between control females and males. Controls, both large and small, and KT treated animals had the lowest KT levels (ANOVA  $F_{7,40} = 12.52$ ,  $p < 0.001$ ), but not significantly lower than the large and small T implanted fish (Tukey's HSD). There was a slight, but significant positive correlation between E2 and aromatase activity ( $R^2 = 0.1$ ,  $p = 0.03$ ).

### **Discussion**

The dominance relationship within each pair of fish determined the expression of behavior, regardless of hormone manipulation. Dominant fish were more aggressive and displayed male courtship behavior, while subordinate fish displayed submissive behavior. In all cases the individual under permissive conditions became social dominants. Physiologically the hormone treatments had significant effects, estrogen treatment prevented morphological sex change in dominant individuals, while KT was able to induce somatic sex change in subordinates. Neither hormone was able to inhibit or facilitate behavioral sex change.

Estrogen treatment inhibits the gonad and external genitalia from becoming male under permissive conditions, while KT masculinizes both of those traits. Across all treatments there was not an effect of absolute levels of estrogen on papilla or gonadal morphology, while KT levels showed a positive linear relationship, across treatment groups, with the degree of masculinization of both the internal and external sexual structures. However, masculinization did not correlate with circulating levels of KT in control animals. This suggests that under natural conditions, changes in circulating KT do not drive protogynous sex change, and further, if KT is involved in the process it is via a change, at the level of the reproductive tissue, in sensitivity to already available KT.

These data generally support the model proposed by Nakamura et al. (2003), that a drop in estrogen is required to allow physiological sex change to proceed, with the masculinizing effects of KT following. In light

of the relationship between KT and masculinization, it is interesting that there were no differences in KT levels between females and newly sex-changed males, similar to previous studies in this species (Rodgers et al., 2006) and in another bi-directional goby (Kroon et al., 2005).

MT moderately masculinizes subordinate fish, most likely through direct effects, as MT fish do not show elevated KT, nor suppressed E2 in this species (as suggested by Nakamura 2003).

Steroid hormones do not regulate behavioral phenotype, or transitions between the male and female states. Estrogen treatment had no effect on aggressive behavior or the expression of male courtship behavior. It is known that changes in aggression can alter brain aromatase levels (Black et al., 2005a), and aromatase regulation can play a key role in somatic sex change (Kroon et al., 2005), but changes in aromatase appear to be a consequence of behavioral changes rather than a mediating force. While aggression can drive down aromatase (Black et al., 2005a), and there is evidence that aromatase can regulate sex change (Kroon et al., 2005), two lines of evidence suggest that this proposed mechanism may not be the only initiator signal from brain to gonad to initiate sex change; while aggression is often associated with sex change (Black et al., 2005b; Reavis and Grober, 1999), changes in aggression are not required for an individual to initiate sex change (Rodgers et al., 2005) and control individuals in this study did not exhibit significant differences in brain aromatase activity. This does not preclude rapid transient effects on aromatase, which were not measurable under this experimental paradigm.

Acquisition of male/dominant typical behavior was expressed independently from levels of steroid hormones, and these behavioral changes resulted in a rapid decline of circulating estrogen. While it may be via a decline in aromatization in some species (Bhandari et al., 2005; Bhandari et al., 2004; Higa et al., 2003; Kroon et al., 2005; Nakamura et al., 2003), it is unlikely to be the mediator in this species, as gonadal aromatase remains elevated (female like) through the early phases of sex change (Black et al., 2005a) and brain aromatase

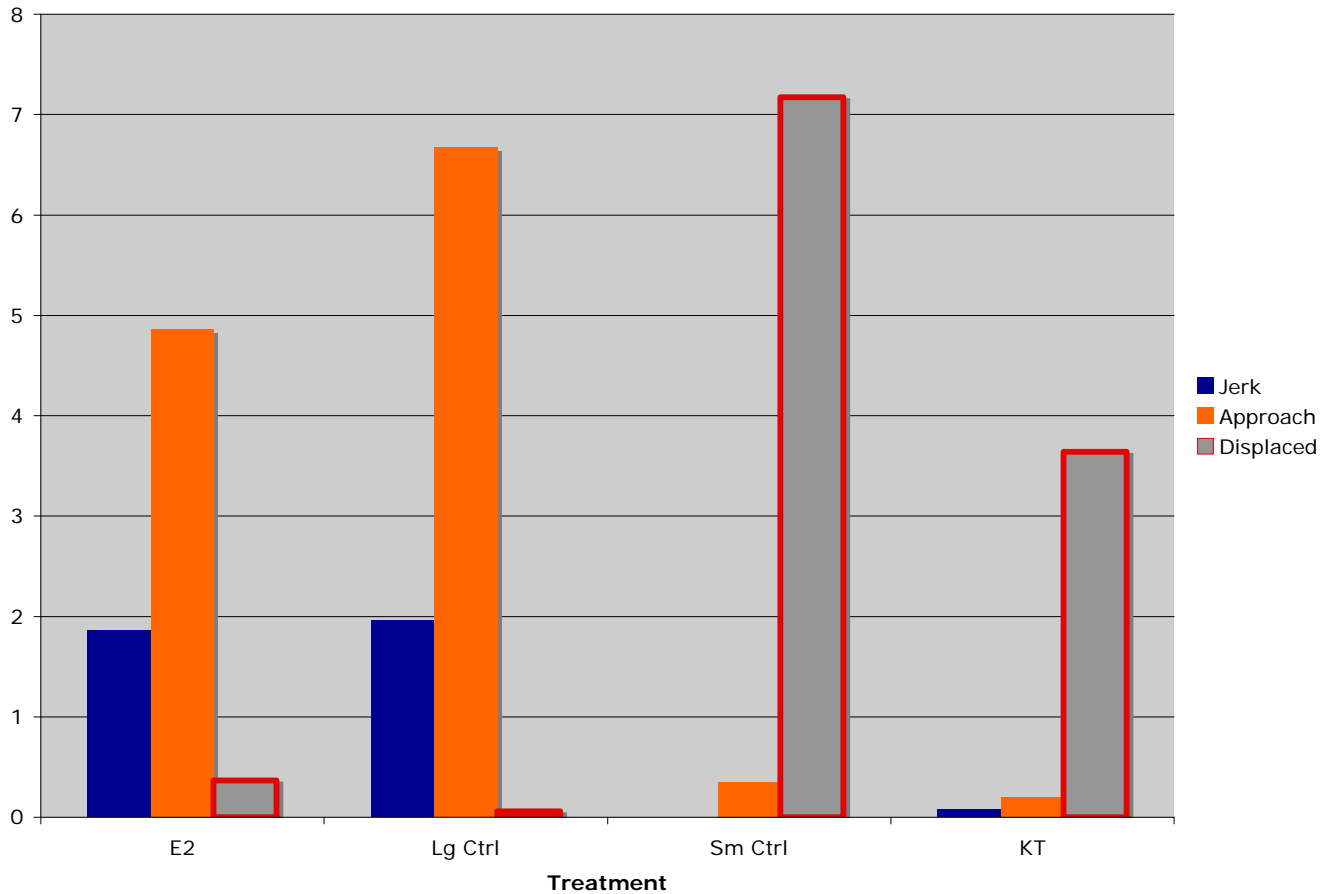
did not show significant differences in the present study. Alternative modes of non-aromatase dependent synthesis of estrogens has been suggested (Ohta et al., 2001), which may account for this apparent discrepancy.

Decision making processes in sexually plastic species have garnered significant interest, with a fundamental question being, how does a fish know when to change sex? Two alternatives have been put forward in the literature; first that the individual is evaluating its own future and current RS as a male and as a female respectively and allocating accordingly (Munoz and Warner, 2003), or second that the individual responds to its own social status, while being blind to the fact that the consequences of changing status can lead to a change in functional sex (Rodgers et al., 2007). A simple experiment can allow the falsification of one of these hypotheses: take the example of a pair of fish, both female, one significantly larger than the other, but neither the size of even an average male. When placed together, which individual, if any, changes sex? This essential question can be illuminated by the control condition in this experiment. If each individual is attending to its own current and future RS, then the large fish should remain female and the smaller fish should change sex, as this provides for the highest RS for both individuals, as predicted by Munoz and Warner (2003). This results from the larger fish being able to produce more eggs, which in this context would be the limiting factor. If the individual is simply attempting to achieve dominance and is blind to changes in status resulting in sex change, the larger fish would change sex against its reproductive best interest. The results from this study again demonstrate that the relevant data stream for decision-making in this species is social status, as the larger fish always changes under those conditions. It further reveals that in this species the individual is blind to the consequences of the decision, meaning that an individual attempts to move up in the dominance hierarchy when an opportunity to interact with a conspecific presents itself, irrespective of how those interactions affect its functional sex and future reproductive success as another sex. The critical result from the empirical side is that in size asymmetric pairs of fish the largest fish became dominant and changed sex into male. If the individual was attending to an RS based cue, as suggested by Munoz and Warner, then the largest individual should have

remained female and the smaller should have changed sex. Thus we can confidently falsify the ERST model in this species.

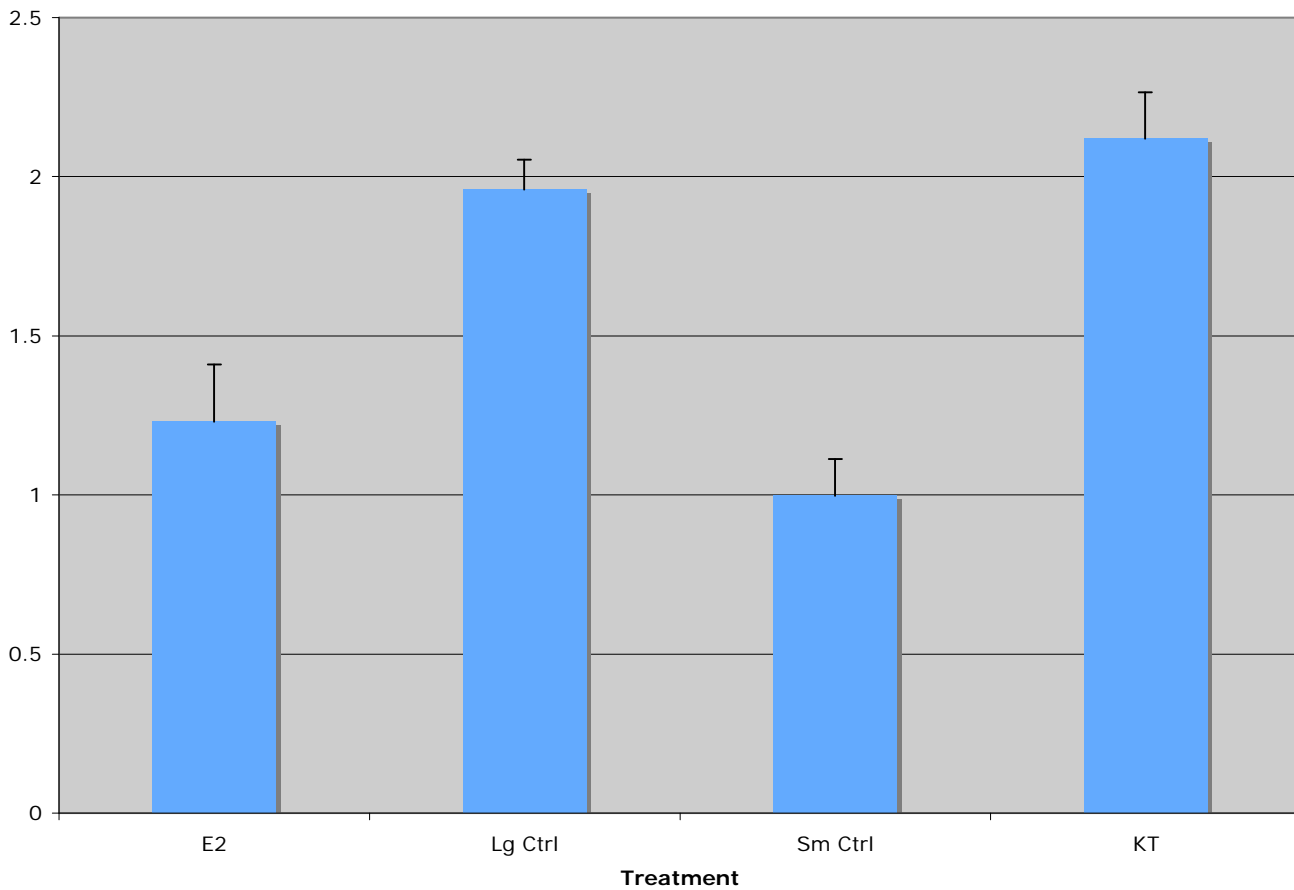
Bi-directional sex changing fish may be under an interesting hormonal constraint with respect to KT, specifically in regard to its relationship to secondary sexual characteristics. Bi-directional fish tend not to exhibit dramatic sexual dimorphism. This likely allows for easier sex change in either direction as conditions warrant, and mechanistically this may preclude dramatic differences in circulating KT levels. This suggests that bi-directional potential may be present in many species, thought to be unidirectional, that do not exhibit dimorphic KT levels or morphology. Bi-directional potential appears to more prevalent than first believed (Kuwamura and Nakashima, 1998; Kuwamura et al., 2002).

Incorporating sex behavior into the examination of the role of steroid hormones in the regulation of sex change will yield greater acuity in understanding a process that incorporates a multitude of coordinated behavioral and physiological changes. The dynamic interactions between the brain, behavior, and gonad, are mediated in some instances through steroid hormones and in other cases likely not.



**Figure 4.1:** Rates of behavior for 4 treatment groups E2, Large control, small control, and KT. Behaviors associated with dominance (aggressive approaches and male courtship) are shown in orange and blue, while subordinate behavior is shown in scarlet and gray. E2 groups were physiologically inhibited from changing sex under permissive conditions, but their behavior is similar to control sex-changing fish. Large controls, which changed sex to male, show aggression and courtship behavior without high rates of submissive behavior. Small controls exhibit predominantly submissive behavior. KT animals were physiologically masculinized, but were behaviorally inhibited.





**Figure 4.2:** Papilla ratio (length/ width) for 4 treatment groups. Female-like ratio is  $\sim 1.0$ , whereas a male-like ratio is greater than 1.5. Large controls and KT treated animals are in the same statistical group while E2 and small controls are in the same statistical group. Note that the E2 treatment was under permissive conditions, showing the inhibitory effects of E2, while KT masculinizes the external genitalia under inhibitory social conditions.

## Chapter 5

### Dmrt1 expression during socially induced protogynous sex reversal

#### **Abstract**

The molecular mechanisms underlying adult sexual plasticity are only beginning to be understood. Recent studies have suggested that life long sexual plasticity has evolved to entrain conserved developmental differentiation pathways to novel regulators. *dmrt1*, a gene involved universally in testis differentiation in vertebrates, is an ideal candidate to be differentially regulated during adult sex change. We characterized the expression of *dmrt1* using quantitative PCR during natural protogynous sex change in the bluebanded goby (*Lythrypnus dalli*) in females, 3 day transitionals, 7 day transitionals, and males. We found *dmrt1* mRNA in testis, ovary, and liver. Expression was higher in testis than ovary, and increased more than two fold over female levels 3 days after the start of protogynous sex change. Transitional animals, both 3 and 7 day, had expression levels above females or control males. The rapid upregulation of *dmrt1* suggests that it is critical for adult female to male sex change, and further supports a model of sexually plastic species utilizing conserved developmental differentiation pathways in adulthood.

## Introduction

Sexual reproduction is nearly universal among animal species, and thus at least two functionally distinct sexes are required. The sexes tend to be segregated into individuals that produce large resource rich gametes and those that produce small abundant gametes. The genetic factors that determine sex are highly variable (Zarkower, 2001), but downstream mechanisms appear to be quite conserved (Marin and Baker, 1998; Morrish and Sinclair, 2002; Wilkins, 1995).

Sex is determined through one of two general mechanisms: genetic factors (e.g. mammals and birds) or environmental factors. Sex determination often occurs early in development, at which time the individual's sexual phenotype is fixed for life. However, this is not the case for all species. Mollusks, crustaceans, echinoderms, polychaete worms, and teleost fishes maintain various degrees of sexual plasticity into adulthood (Ghiselin, 1969; Policansky, 1982). This plasticity, under appropriate conditions, can lead to dramatic increases in reproductive success (Ghiselin, 1969). A large group of advanced fishes, the teleosts, exhibit a stunning variety of flexible approaches to sexual differentiation that range from alternative sexual phenotypes within a sex, to functional adult sex change (Devlin and Nagahama, 2002; Kuwamura and Nakashima, 1998).

Recent work in the bluebanded goby (*Lythrypnus dalli*) led to the proposal of a model of the evolution of sex change whereby the conserved molecular pathways for sexual differentiation have become entrained to a cue that varies over the life history of an animal (Rodgers et al., 2007). This variable signal model (VSM) proposes that in adult sexual differentiation (i.e. re-differentiation), a novel regulator usurped control of conserved developmental cascades, similar to the retrograde pathway evolution mechanisms proposed by Wilkens (1995). In the bluebanded goby, the cue that regulates sexual transformations is likely to be a neurochemical representation of status (Chapter 3). To examine the potential utility of this model, identification and characterization of genes within these conserved modules is necessary. The DM gene family and particularly *dmrt1* is an ideal candidate for reasons detailed below.

Although upstream regulators of sex vary considerably, the DM family of transcription factors regulate sexual differentiation in wide range of species, including corals, nematodes, insects, and humans. These zinc-finger transcription factors were first described in *Drosophila*, as an alternatively spliced gene, double-sex (*dsx*), that determine sex in males through expression of *DSX<sup>m</sup>*, and in females through expression of *DSX<sup>f</sup>* (Burtis and Baker, 1989). In *C. elegans*, *mab-3* and *mab-23* are critically involved in production of the male sex (Hodgkin, 2002; Raymond et al., 1998; Shen and Hodgkin, 1988). These genes share high sequence similarity in a region that named the DM domain (for *dsx* and *mab-3*) that also has similar functionality (Raymond et al., 1998; Yi and Zarkower, 1999). Ectopic expression of *DSX<sup>m</sup>* can partially rescue *Mab-3* mutants (Raymond et al., 1998).

A human testis determining gene, *dmrt 1*, was identified as the vertebrate homologue of *dsx* and *mab3* (Raymond et al., 1998). Subsequent examination in the mouse and chicken showed high expression in the genital ridge prior to sexual differentiation, and more robustly in males than in females (Raymond et al., 1999). *dmrt1* has been cloned in a number of vertebrates including mammals (De Grandi et al., 2000; Pask et al., 2003; Smith et al., 1999), birds (Shan et al., 2000; Shetty et al., 2002; Smith et al., 1999), reptiles (Kettlewell et al., 2000; Murdock and Wibbels, 2006; Shoemaker et al., 2007; Smith et al., 1999), and fish (Guan et al., 2000; Huang et al., 2002; Kobayashi et al., 2004; Matsuda et al., 2002; Xia et al., 2007).

DM-domain containing genes are involved in sexual development from corals (Miller et al., 2003) to humans (Volff et al., 2003). *Dmrt1* knockout mice do not form functional testis (Raymond et al., 2000), and expression of *dmrt1* or its paralogue *DMY* can induce sex-reversal in genetic females (Matsuda et al., 2007; Smith et al., 2003). *Dmrt1* orthologues share a highly conserved DM domain and varying degrees of similarity thereafter (Zhu et al., 2000) and are more homologous to each other than to other DM paralogues (Volff et al., 2003).

To date, only one study has examined *dmrt1* expression in a sexually plastic teleost species (Xia et al., 2007). Examining two species of protogynous grouper, Xia et al. (2007) found differences in *dmrt1* gene expression between males and females, and were able to induce sex reversal and *dmrt1* expression in females via administration of exogenous alpha-methyltestosterone (MT). The present study takes advantage of several features of *L. dalli*, which make it more tractable to examine changes in this gene over the course of natural sex reversal. First, protogynous sex change is readily induced through changes in social environment. Sex change occurs rapidly (in about 2 weeks) in the dominant female after removal of the male, in the field (Black et al., 2005b) and in the laboratory (Reavis and Grober, 1999). This allows for characterization of *dmrt1* over the course of natural sex change. In *L. dalli*, the gonad can be masculinized through exogenous androgen treatment (Carlisle et al., 2000), but there are no differences between the sexes in androgen levels (Rodgers et al., 2006). The VSM posits that lifelong sexual plasticity is generated by linking the conserved developmental pathways of sexual differentiation to regulators that vary over the life history of the animal. *dmrt1* is a prominent member of these conserved developmental cascades, and as such we hypothesize that *dmrt1* is elevated in males compared to females and that its expression increases significantly over the course of protogynous sex change.

## Materials and Methods

### *Experimental Design*

Partial *dmrt1* sequence was identified via a degenerate PCR based approach, and then gonadal *dmrt1* expression was measured using quantitative PCR at four time points: female, 3 day transitional, 7 day transitional, and male. Twenty social groups were established and allowed to spawn. After all the groups had spawned, the males were removed, 10 of which were collected for the male sample. The dominant animal was collected three days after male removal in 10 groups and 7 days after male removal in the other 10 groups. Ten females were taken randomly, either at the time of initial male removal, or another subordinate female was

taken from transitional groups. Using these sampling points (female, 3 day transitional, 7 day transitional, and male), we analyzed *dmrt1* expression profiles over the early course of natural sex change in the bluebanded goby. Sex was determined by visual examination of the genital papilla under a dissecting scope. Females possess a blunt, square shaped papilla with a length to width ratio of approximately 1.0, males have a elongated and pointed papilla that typically has a ratio of 1.6 or greater (St. Mary, 1994). Individuals with ambiguous papillae were not introduced to the study groups. Fish were collected (CF & G permit # 803034-01) on Santa Catalina Island, California, using an anesthetic solution of quinaldine sulfate (Sigma Chemical) and hand-nets. Animals were housed at the Wrigley Marine Science Center (Santa Catalina Island), in flow-through sea water tables. Animals were kept under natural light conditions. At the above noted time points, animals were euthanized using an overdose of MS222. The gonads, liver, and brains were removed, stored in *RNAlater* (Ambion), and transported to Georgia State University. This research was carried out in accordance with the IACUC standards for use of animals in research at University of Southern California and Georgia State University.

#### *RNA isolation*

Total RNA was isolated from gonad, liver, and brain (50 mg per sample tissue type, pooled sample) using TRIZOL reagent (Invitrogen), following manufacturer recommendations. mRNA was isolated from total RNA using MicroPoly A Purist (Ambion), following the manufacturers protocol.

#### *Degenerate RT-PCR*

Reverse transcription was performed using Superscript III (Invitrogen) with oligo (dT)<sub>20</sub> primers using 100 ng of mRNA in 20 µl reactions. The primers listed below were then used in standard degenerate PCRs to isolate 595 bp of the *L. dalli dmrt1* gene. Initially, a 120 bp fragment of *L. dalli dmrt1* was amplified using primers from Raymond *et al.* (1999), which have been used to clone the gene in zebrafish and rice eel (Huang *et al.*, 2002), and mouse (DM F1 and DM R1). To extend the 120 bp fragment, an *L. dalli* forward primer was

paired with a reverse degenerate primer (Ld DM F1 and DM R2). Based on the resulting sequence, an *L. dalli* specific reverse primer was made for quantification (LD R3). A 250 bp fragment of the *L. dalli* glyceraldehyde-3-phosphate dehydrogenase (GAPDH) gene was also obtained using the degenerate primers shown below.

DM F1: 5'-TGCGCVMGRTGCMGRAATCACGG-3'

DM R1: 5'-CKSAGSGCSACCTGSGCCGCCAT-3'

Ld DM F1: 5'-GTGTCCCAAGTGCAAGCTCATCG-3'

DM R2: 5'-GGTAGGTGGCWGCTGGGTAGTA-3'

Ld DM R3: 5'-CCATGAGGCATCTGGTACTGCTGGTAGTTGTA-3'

GAPDH F1: 5'-TGCCAAGGCTGTBGGCAARGT-3'

GAPDH R1: 5'-CGCCRGCATCAAAGATGGAGGA-3'

PCR cycling conditions were as follows: 40 cycles, 94°C 1 min, anneal at 52°C 1 min, 72°C 1 min. All reactions contained 1 µl template cDNA, with 0.5 µl forward and reverse primer (10 mM) with 23 µl platinum taq supermix (Invitrogen). PCRs were run on 2% agarose gels and stained with ethidium bromide. Resulting bands were excised from the gel and purified using PureLink Quick gel extraction kit (Invitrogen). Following purification, sample PCR products for each tissue and primer set were sequenced by the Georgia State University Molecular Core Facility. Sequencing results were analyzed using a BLAST search (NCBI) for *dmrt1* and sequence alignments (Vector NTI, Invitrogen).

#### *Generation of an external standard*

To quantify changes in *dmrt 1* expression in the background of a re-organizing tissue, we generated an external standard with which to normalize *dmrt 1* expression levels across samples, as described in Baro et al. (1997). The external standard represents a rearrangement of the endogenous *L. dalli dmrt 1* such that the primers used to amplify the endogenous *dmrt 1* gene will also recognize and amplify the standard, but produce a different size product. Thus, when the endogenous standard is added to mRNA and an RT-PCR is performed,

the PCR products resulting from the endogenous transcripts and the external standard can be separated by size using gel electrophoresis.

The external standard was made by a deleting approximately 100 bp of a 393 bp *dmrt1* clone and inserting xbp of pCR2 vector. First, a 393 bp fragment of *dmrt1* was cloned using a TA Cloning kit (Invitrogen), following the manufacturer's instructions. Fortuitously, one clone contained a truncated insert due to bacterial recombination. This clone was missing the last 100 bp of the 3' end of the insert. Next, SacI and KpnI sites were added to the reverse primer described above, and it was ligated into the truncated colony. Finally, this clone was used as a template in an *in vitro* transcription reaction. The plasmid was linearized using HindIII in a standard restriction digest and transcribed into mRNA using the mMESSAGE mMACHINE T7 Ultra kit (Ambion), per manufacturer's instructions. The resulting RNA was then quantified and used as a standard in quantitative RT-PCR experiments.

#### *Quantitative RT-PCR*

Sample mRNA was isolated from tissue as described above. Five ng of each sample was combined with xug of the external RNA standard. RNA was reverse transcribed into cDNA in a 10 µl RT reaction as described above using gene specific reverse primer Ld DM R3. The entire RT was then used as a template in a PCR. Thirty-nine µl of (taq plus mix to be named later) and 0.5 µl (10 mM) of each primer DM F1 and Ld DM R3 were added to the RT reaction and run for 35 cycles, using the same parameters described above. PCR products were then run on 10% polyacrylamide gel. The gel was imaged and the optical densities (ODs) of the two product bands (i.e., *dmrt1* and external standard) were measured using a Fluorchem imager and Alpha Innotech software package. Dividing the OD of *dmrt1* by that of the external standard normalized expression. Normalized *dmrt1* expression could then be compared across samples.

For the quantitation to be valid, both the endogenous and external standard signals must remain within the linear range of amplification (Baro et al., 1997). The linear range of amplification was determined by



running various concentrations of RNA external standard (Figure 5.1) with 5 ng of tissue for 35 cycles under the same conditions described above. Samples were again run on polyacrylamide gels and stained with ethidium bromide. These results produced a standard curve (Fig. 3), and an appropriate concentration was selected ( $7 \times 10^5$ ) and used for the experiment.

## Results

To examine sequence similarity between *dmrt1* in *L. dalli* and other species, we sequenced 595 bp of the *L. dalli dmrt1* and compared that sequence to existing *dmrt1* sequences. *L. dalli* had 91% sequence similarity within the DM domain of the grouper (ascension #) and an overall similarity of 76%. The sequence did not BLAST or align with other DM family genes from fishes or mammals. RT-PCR was used to determine that *dmrt1* is expressed in the testis, ovary, and liver of *L. dalli*.

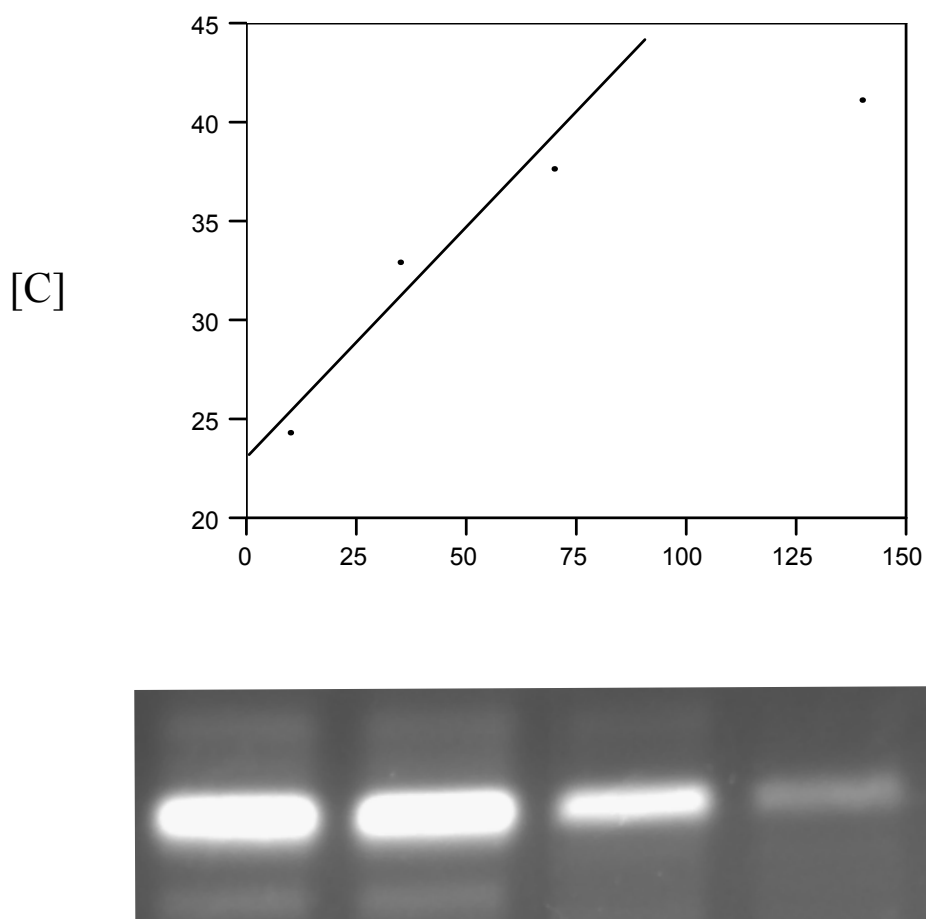
Quantitative analysis shows a dramatic up-regulation of *dmrt1* in the transitional gonad, as early as day 3 after male removal. Expression more than doubles during sex change, but is lower in established males. *Dmrt1* expression is approximately 50% higher in males than in control females (Figure 5.2). Liver expression did not change over sexual transition.

## Discussion

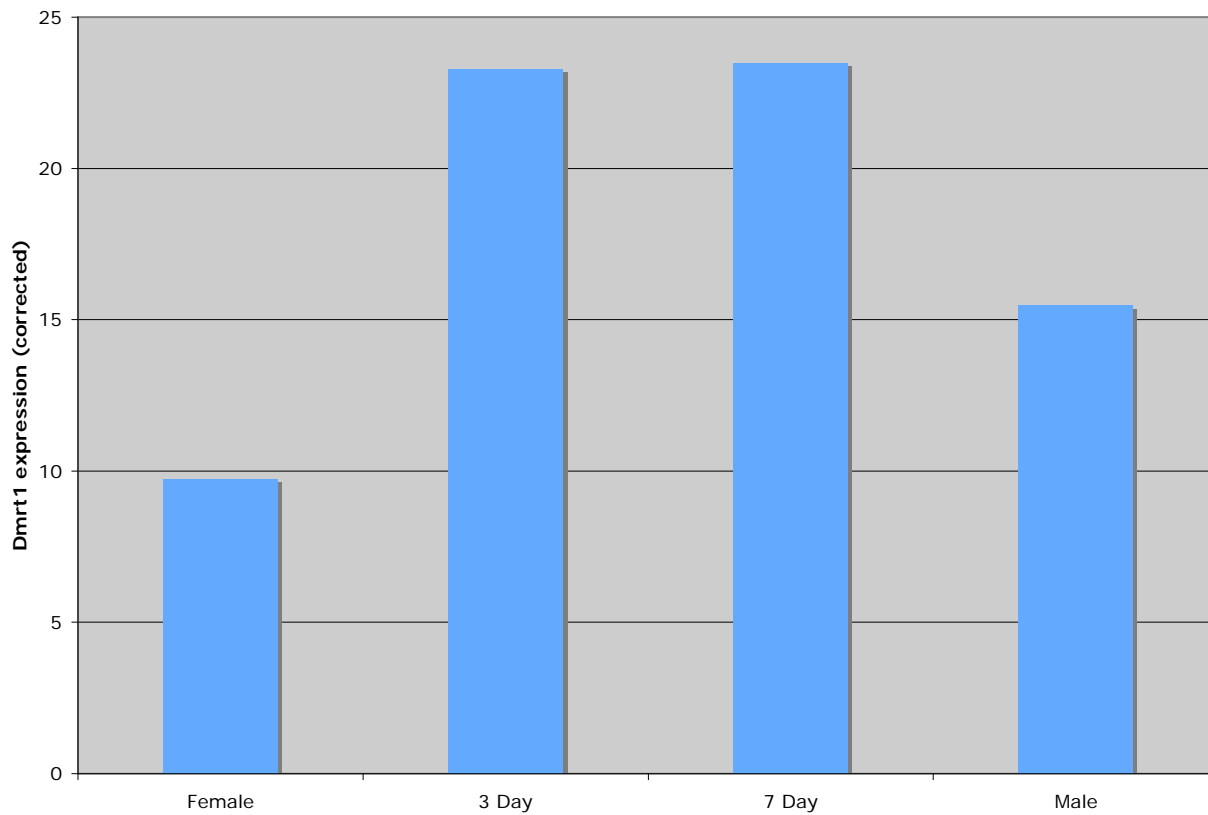
*Dmrt1* expression was identified in the gonads of males and females as well as in the liver. This pattern is similar to that of zebrafish Gao et al (2005). BLAST search indicated unequivocally the gene was *L. dalli dmrt1*. It has high sequence similarity with grouper *dmrt1* (76% sequence identity) and stickleback (81% sequence identity), and did not identify with non-*dmrt1* DM gene family genes. GAPDH was expressed in all tissues examined.

Gene expression increases dramatically from the female baseline by 3 days after male removal. Expression remains elevated at the 7-day time point, before declining somewhat to the established male level. Males have about 50% more expression than females. Transitional animals have more than double the expression of females and 50% greater than established males. The rapid up-regulation of *dmrt1* suggests that it is play a role in organ remodeling and testis differentiation. This is consistent with the idea of re-differentiation described by the variable signal model; in early development *dmrt1* is expressed in the genital ridge of mice and chickens (Raymond et al., 1999). As a control tissue, liver expression was not sexually dimorphic, nor did it change over the course of sex change. This reveals that the dimorphic expression patterns are restricted to the gonad, and changes in expression correlate with sex change, broadly speaking.

Elevation of *dmrt1* by day 3 reveals that the signal to change sex reaches the gonad in short order. The signal, already having reached a plateau by day 3, must arrive even sooner, suggesting that the decision to engage these developmental pathways must be rapidly executed following a change in status. It will be interesting to discover whether only the alpha individual (i.e., the sex changer) increases expression, as would be predicted, or whether other individuals in the group do as well in response to social instability. Further characterization of this gene will enable a better understanding of how rapidly the signal to change sex arrives from the brain following a change in social status. The next step will necessarily involve localizing within the gonad where *dmrt1* is being expressed, via *in situ* hybridization, and how that maps on to gonadal re-organization during sex change.



**Figure 5.1:** Standard curve for the control gene. Y-axis represents concentration,  $\times 10^5$  molecules, and expression intensity on the x-axis. The final two concentrations reached saturation. Concentration x was chosen for the experiment. Concentrations were derived from three independent serial dilutions.



**Figure 5.3:** Dmrt1 expression in the gonad across sex change. Females and males show dmrt1 expression, which is 50% higher in males. Dmrt1 is rapidly upregulated upon male removal, within 3 days expression more than doubles. It remains elevated at 7 days, and is lower in full breeding males.

## General Discussion

This series of studies addressed the phenomenon of adult sexual plasticity on several levels of analysis, from behavioral regulation to evolutionary models. A model at the proximate level is depicted in Figure 6.1. From the perspective of the individual, interactions with the social environment are used to establish social status. Sexual phenotype is then brought into concordance with social status. In the case of female-to-male sex change, that process involves an elevation of social status, a necessary decrease in circulating estrogens and an increase in gonadal *dmrt1* expression. These physiological effects are theorized to occur within limited gonadal space, meaning the individual must take down one tissue type to build the opposite.

To better understand the evolution of sexual plasticity in fishes, we have proposed the variable signal model (VSM), which posits that functional sex change has evolved to entrain conserved developmental pathways to a signal that varies over the life history of the individual. In many ecological situations, social status is uniquely suited to fill the role of that signal, owing to 1) its pre-existing relationship with RS, 2) the fact that changes in status are predictive of changes in reproductive opportunity, and 3) the fact that status is relatively stable in many animal species. Thus in species with strict hierarchies, individuals tend to be subordinate when small and only have an opportunity for dominance when they grow large enough to vie for top status. A dynamic cue, such as social status, gives the individual greater flexibility than a fixed size or time point for sexual transition and would certainly out-compete such a strategy.

The relative contributions of the previous chapters to these synthetic models are discussed in the following sections, in addition to their relationship with existing theories and models for understanding sex change.

### *Size advantage*

The convention for understanding sequential hermaphroditism has long been the size advantage model. This model predicts the potential for and the direction of sex change, under different ecological conditions, and has

been a useful framework for understanding “why” sequential hermaphroditism is selectively advantageous. The question of “how” a decision is made to change sex or not, does not appear in the original model (Ghiselin, 1969; Warner et al., 1975). Extensions of this model to the proximate level, to address this question, have proved difficult, although attempts have been made (Munday et al., 2006; Munoz and Warner, 2003). Two alternatives have been proposed. The first is that the individual evaluates its own future and current RS as a male and as a female respectively and allocates accordingly (Munoz and Warner, 2003). The second proposal is that the individual responds to its own social status, while being blind to the fact that a key consequence of changing status can be a change in functional sex (Chapter 3). The following will highlight the contributions of my dissertation research to these questions.

Empirical data demonstrating variation among populations in the size at sex change generated controversy (Shapiro, 1987; Shapiro, 1988) over the original formulation of Warner’s model (1975), which was based on a genetic mechanism. This led to a revision that placed relative size, as opposed to absolute size, as the critical variable. Relative size is certainly a more dynamic approach, but it requires an accurate assessment mechanism and a decision matrix. Such an extension to the proximate level allows for direct empirical testing. If in fact, animals assess relative size, how are these decisions made when size asymmetries are minimized? This was an essential question dealt with in Chapter 1. The results show that within female dyads introduced into a neutral environment, behavioral interactions determine which individual will change to male and which will stay female. Chapter 1 also shows that small size differences are not predictive of which individual will change sex. These results demonstrated that *L. dalli* can use behavioral cues, independent of size information, to determine sexual phenotype. These results alone demonstrate that size is not being evaluated as a proximate cue in *L. dalli*. Moreover, if size is used when asymmetries are easily discernable, then it would be through a second independent assessment mechanism. Considering that behavioral interactions work as an effective cue over all ranges of sizes, whereas size ceases to work when asymmetries are minimized, parsimony requires that

we opt for a single mechanism that explains all of the data rather than invoking two independent mechanisms. In sum, status, not directly size, provides the critical information stream used to determine whether or not an individual changes sex. This suggests that the association between size and sex change is an epiphenomenon related to the effects of size on the determination of social status, rather than any direct size based mechanism.

The size advantage model works well for protogynous (female to male) and protandrous (male to female) sex change, but does not easily accommodate species in which individuals change sex in both directions (Kuwamura and Nakashima, 1998; Kuwamura et al., 2002; Munday, 2002; Sunobe and Nakazono, 1993). This is because size advantage predicts a directionality of selection based on potential reproductive success, such as on female to male sex change when an animal reaches a certain size or age. But size advantage does not predict selection on a later transition from a sexual phenotype with higher predicted fecundity (male) to one with lower predicted fecundity (female), or species that exhibit sexual plasticity in the absence of a dramatic size/fecundity skew. The application of the size advantage model to a species such as *L. dalli* predicts protogyny, because a large male can monopolize the reproductive potential of several females. However, like other species exhibiting a harem social structure, *L. dalli* shows bi-directional sex change. The other major model to explain bi-directional sex change, the growth rate advantage model (Kuwamura et al., 1994), is not applicable to this species, since males grow faster than females (Chapter 1), in contrast to those species where growth rate advantage has purchase.

The results of Chapter 3 demonstrate that a proximate mechanism based on social status can regulate sex change in both directions, in bi-directional harem species and in pair living species. This study solidified social status as the proximate cue utilized in making sex allocation decisions in the bluebanded goby. The striking parallelism demonstrated in both directions suggested a new model system to better understand the evolution and proximate regulation of sexual plasticity in fishes, the VSM. Because this model focuses on the specific proximate factors that drive the evolution of sex change, it can be used explain the persistence of sexual

plasticity across a range of species and mating systems that are not subject to dramatic size-fecundity skew (Kuwamura et al., 1994) but where phenotypic flexibility would be reproductively advantageous, such as after mate loss or social disruption in pair-living populations (Munday et al., 1998; Nakashima et al., 1995; Sunobe and Nakazono, 1993). The latter condition would be most similar to the low density model originally described by Ghiselen (1969).

Chapter 4 reduced the complexity of the two competing models, ERST versus a status-based version of the VSM, to a simple experiment that can allow the falsification of one of these hypotheses. Take as an example a pair of fish, both female, one significantly larger than the other, but neither the size of even an average male. When placed together, which individual, if any, changes sex? The results from this study again demonstrate that the relevant data stream for decision-making in this species is social status, as the larger fish always changes. It further supports the hypothesis that in this species the individual is blind to the consequences of the decision, meaning that an individual attempts to move up in the dominance hierarchy when an opportunity to interact with a conspecific presents itself, irrespective of how those interactions affect its functional sex. The critical result from the empirical side is that in size asymmetric pairs of fish the largest fish became dominant and changed sex into male. If the individual were attending to an RS based cue, as suggested by Munõz and Warner, then the largest individual should have remained female and the smaller should have changed sex. Thus we can confidently falsify the ERST model in this species. This situation is likely to be the case for most species of sexually plastic fishes, and we predict that rigorous testing of the model will demonstrate this. A more encompassing model, such as the VSM or other models that emphasize how fish make life history decisions, with an eye toward mechanisms and levels of selection, will be more useful as an organizing principle for the field.



*Androgens, aggression, and paternal care*

In *L. dalli*, elevated levels of KT are not incompatible with paternal behavior (Chapter 2). In species where a high degree of male parental care is required for successful rearing of offspring, insensitivity to testosterone has been observed (Hunt et al., 1997; Lynn et al., 2002; Lynn et al., 2005; Van Duyse et al., 2000). Paternal care is required in *L. dalli* for successful hatching. However, the observed increase in KT while males are actively parenting suggests that KT is positively associated with paternal care in experienced males. While this does not conform to the classic trade-off model of androgens in parenting males, it is consistent with evidence from a number of studies involving fish (Kindler et al., 1991; Ros et al., 2004) and rodents (Trainor and Marler, 2001), where it has been demonstrated that androgens either do not interfere with, or potentially facilitate, paternal care (Kramer, 1972; Trainor and Marler, 2002). In cotton-top tamarins, parental experience has differential effects on male testosterone responses to female pregnancy, with more experienced males showing greater elevation of testosterone than less experienced males (Ziegler et al., 2004). Recent work in the bluegill sunfish has shown that androgens do not interfere with paternal care and that males in the best condition maintained high androgens throughout the parenting phase (Magee et al., 2006).

In many species, territorial behavior and courtship are temporally disassociated from parental care, whereas in *L. dalli* these behavioral suites occur in concert. A positive correlation between androgens and paternal care in species that must simultaneously defend a nest, court females, spawn, and care for offspring has been predicted by Marler et al. (2003), and our findings in *L. dalli* clearly support their prediction.

There are several possibilities for why KT may be elevated during parenting. The first is that KT is positively associated with elevated aggression (Ros et al., 2004) and males may need to raise their level of aggression to adequately defend the nest. While we did not examine rates of aggressive behavior in this study, it is known that *L. dalli* males exclude females, and potentially other males, from the nest regardless of presence of eggs (Black et al., 2005b; Rodgers et al., 2005). A male's need to constantly defend the nest should not

interfere with his ability to parent appropriately in species that must do both simultaneously. *L. dalli* males continue to court and spawn with females throughout any given brood cycle, and it may be this courting behavior that requires KT to remain high. In many fish species, KT is high when males are courting and during the early stages of parental care (Knapp et al., 1999; Pall et al., 2002a; Sikkell, 1993). It is reasonable to assume that KT may contribute to high levels of courtship behavior or vice versa. Finally, KT may drive the expression of a suite of parental behaviors (rubbing, nipping, and others) that have a direct positive affect on offspring survival.

#### *Steroid hormones and sexual plasticity*

Status and the agonistic behavior that determines it regulate the expression of sexual phenotype (Chapter 3). Steroid hormones do not affect the behavior involved in status acquisition and thus are likely downstream activators in the sex change cascade (Chapter 4). Estrogen treatment has no effect on the expression of aggressive or male courtship behavior, under socially permissive conditions. It is known that changes in aggression can alter brain aromatase levels (Black et al., 2005a), and aromatase regulation can play a key role in somatic sex change (Kroon et al., 2005). Chapter 4 suggests that changes in aromatase are a consequence of behavioral changes rather than a driving force. Additional support for this is gleaned from KT implanted fish: KT down-regulates brain aromatase activity but fails to elicit behavioral sex change. While aggression can drive down aromatase (Black et al., 2005a), and there is evidence that aromatase can regulate sex change (Kroon et al., 2005), two lines of evidence suggest that this proposed mechanism may not be the only signal from brain to gonad to initiate sex change. The first is that although aggression is often associated with sex change (Black et al., 2005b; Reavis and Grober, 1999), changes in aggression are not required for an individual to initiate sex change (Chapter 1). The second is that control individuals in did not exhibit significant differences in brain aromatase activity (Chapter 3). This does not preclude rapid transient effects on aromatase, which were not measurable under the current experimental paradigm.

Changes in behavior are expressed independently from steroid hormones, and these behavioral changes result in a rapid decline of circulating estrogen (Figure 6.1). While it may be via a decline in aromatization in some species (Bhandari et al., 2005; Bhandari et al., 2004; Higa et al., 2003; Kroon et al., 2005; Nakamura et al., 2003), it is unlikely to be the mediator in this species, as gonadal aromatase remains elevated (female like) through the early phases of sex change (Black et al., 2005a) and brain aromatase does not show significant differences either (Chapter 4). Alternative modes of non-aromatase dependent estrogen synthesis have been suggested (Ohta et al., 2001), which may account for this apparent discrepancy.

Steroid hormones do have an effect on somatic sex, consistent with a large body of literature from other species (Frisch, 2004). Estrogen declines during natural protogynous sex change and artificially elevating estrogen prevents morphological sex change in both the gonad and external genitalia. In contrast to some sex changing species, but certainly not all, KT does not increase significantly during protogynous sex change although KT administration masculinizes both the gonad and external genitalia, and induces the formation of the AGS. Testosterone, while not sexually dimorphic, may have some activity on its own, but it is likely through either conversion into other bioactive steroids (E2 or KT) or through changes in tissue sensitivity, perhaps via up-regulation of the androgen receptor. Circulating E2 levels are important in regulating sexual phenotype, as a decline in E2 is required for the formation of a male gonad, but alteration of circulating androgen levels are not. Androgens are likely to be important, owing to their ability to masculinize female morphology, but this is likely mediated through changes in specific tissue sensitivity to the hormone, as circulating androgens are not dimorphic, while gonadal and genital morphology are.

Taken together, the results from Chapters 2, 3, and 4 suggest that while androgens do have a role in sexual transitions, their actions are downstream and independent of the behavioral interactions and the emergent neurochemical hallmark of status that initiates sexual transformation.

*The Variable Signal Model: towards a synthetic evolutionary model of sexual plasticity*

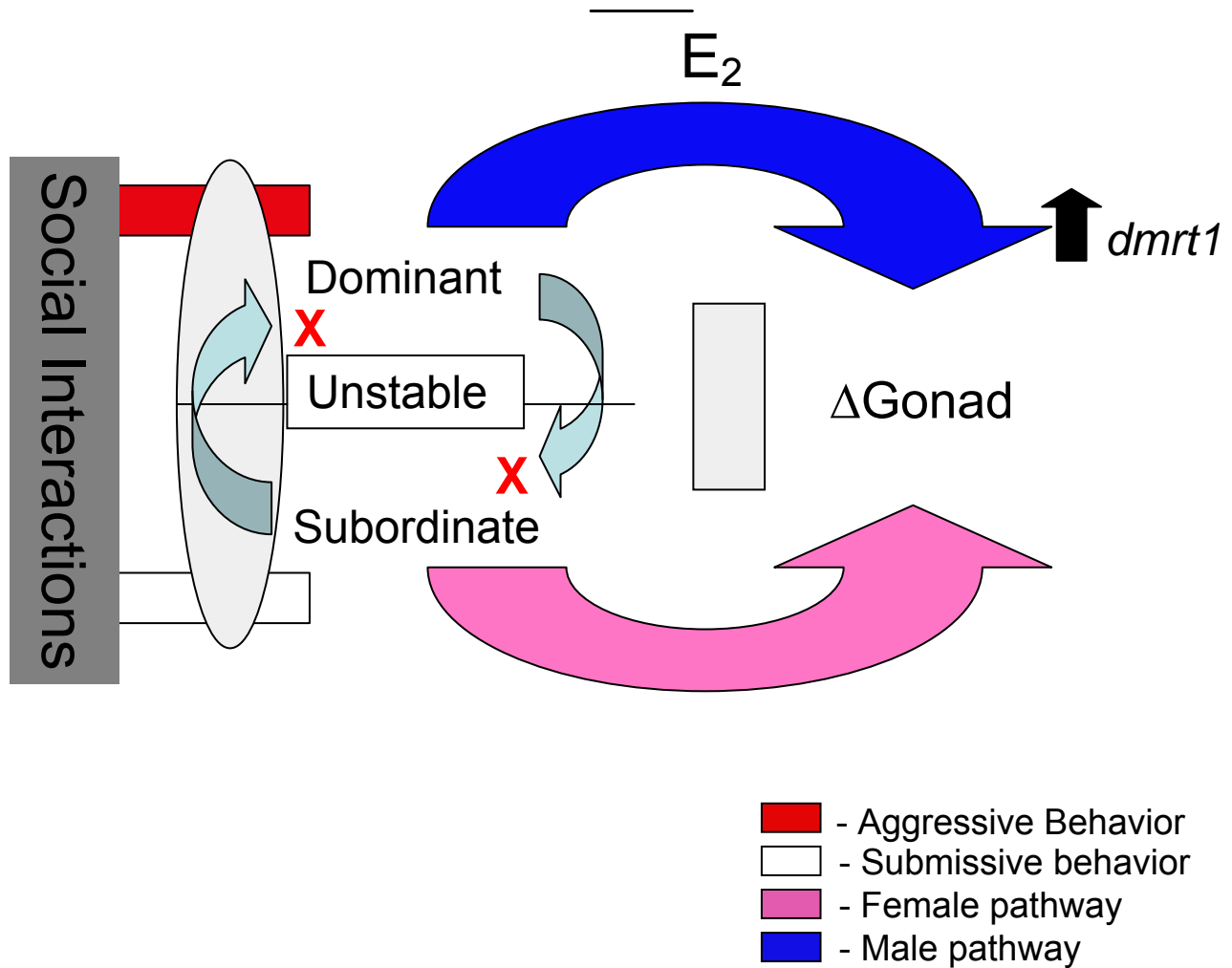
How did sexual plasticity evolve? Why, when the selective pressures making sexual plasticity favorable are so prevalent among animals, do so few species exhibit such plasticity? Do the multiple independent evolutions of sex change share convergent features and if so what are they and why? These are the kinds of questions that, in a post size advantage era, will drive the study and frame our understanding of sexually plastic fishes.

The striking parallelism in Chapter 3 between sex change in the protogynous and protandrous directions with respect to both the time course and the use of social status suggest that a similar mechanism may underlie both processes. The simplest way for such a mechanism to work is to utilize the already existing genetic machinery that builds testes and ovaries in early development, and entrain them to a regulator that is predictive of dynamic changes in reproductive opportunity. Social status provides an ideal signal in that regard, because of a pre-existing relationship between dominance and reproductive success (Ellis, 1995), and because young animals are unlikely to be dominant or to reproduce effectively as the resource holding sex. This would allow for lifelong local reproductive responsiveness, irrespective of absolute size or age. There is a precedent for such a mechanism within this particular system, as throughout evolutionary history, control over sexual differentiation pathways has been entrained to a variety of different signals (Zarkower, 2001), and it has been suggested that adding upstream regulators to a cascade is more likely than changing entire downstream pathways (Wilkins, 1995). Such a mechanism would allow individuals to take advantage of the differences in size-fecundity skew between the sexes originally described in the size advantage model (Ghiselin, 1969; Warner, 1988; Warner et al., 1975). This model proposes that sexual plasticity has evolved by shifting control of sexual differentiation from a static genetic signal to a signal that may vary over the life of an individual, a variable signal. Thus both unidirectional and bi-directional sex-changing fishes are likely to use the conserved

process of sexual differentiation that is now linked to a variable signal (in this case, social status) to direct adult re-allocation of the suite of traits that characterize the sexes.

A test this model requires the identification of genes involved in these conserved developmental cascades. An initial test was carried out in Chapter 5, with the characterization of *dmrt1* in *L. dalli*. The results show that it is expressed in a normal pattern for fishes, and that it does indeed increase over protogynous sex change. This warrants further tests of the model with more rigorous examination of these gene cascades and how they function in adult sex change.

When examining sexual plasticity in light of the VSM, an intriguing prospect emerges: bi-directional sex change is ancestral to unidirectional sex change. The VSM predicts that a regulatory factor, unconstrained by a developmental critical period, has usurped access to the usually fixed developmental pathways that differentiate individuals into one sex or the other. Such a regulator, in order to initiate sex change, must be activated during adulthood. There is no *a priori* reason to suspect that this signal could not be turned off as well, leading to reversion to the original phenotype. It is easily conceivable and entirely likely that subsequent modification, perhaps through sexual selection, would canalize a sexual transition, making it over time, unidirectional. In sexually plastic fishes that exhibit dramatic sexual dimorphism, such as the bluehead wrasse, this is likely the case. Such a mechanism is likely to be more stable than it might appear at first glance, as several ecologically protogynous species have been shown to possess latent bi-directional capacity (Chapter 3 and Kuwamura et al., 2002)). Under the VSM, with its emphasis on the adaptation being plasticity rather than sex change in any specific direction, socio-ecological structure would determine under which conditions change would be advantageous, allowing for sexual plasticity to persist or evolve under conditions that are not subject to size-fecundity skew. The VSM provides a framework for examining sexual plasticity that integrates important aspects of both developmental mechanisms and evolutionary history.



**Figure 6.1:** Integrative model of sex change for the bluebanded goby. Sexual phenotype in both directions are determined by dynamic behavioral interactions, not by size (Chapter 1,3, & 4), and independent of gonadal constitution (Chapter 3). Dynamic social interactions give rise to a status, which, with respect to sexual phenotype, is dichotomized into dominant or subordinate (Chapter 3). Status activates a sex specific pathway (Chapters 4 & 5) and inhibits the behavior not concordant with sexual phenotype, such as egg consumption in dominants (Chapters 1 & 2) and or jerking behavior in subordinates (Chapter 3). Activation of the male specific pathway involves a drop in circulating estrogens (Chapter 4) and an increase in *dmrt1* expression in the gonad (Chapter 5).

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