When social environments are unpredictable, individuals must be able to adapt to these changes by rapidly modifying their behavior. Since these behavioral responses are likely to involve rapid actions of neuropeptides in the brain, understanding these pathways is crucial to an understanding of how animals adapt to their social environment. The bluehead wrasse (*Thalassoma bifasciatum*) is an excellent model system for approaching these questions for many reasons. Primarily socially-controlled female-to-male sex change naturally decouples the brain from gonadal influences and can be exploited to more directly examine other influences, such as social interactions, on the neural substrates of reproductive behavior.

Arginine vasotocin (AVT) and its mammalian homologoue arginine vasopressin (AVP) influence male sexual and aggressive behaviors in many species. To test the effects of AVT on the mediation of male behavior, we gave AVT injections to territorial and non-territorial males of the large and colorful phenotype (terminal phase) and an AVP-V₁ receptor antagonist to territorial males in the field. In territorial males, AVT increased courtship and tended to decrease the number of chases towards initial phase individuals. In non-territorial males, AVT increased courtship, chases towards initial phase individuals, and territorial behavior while decreasing feeding, all behaviors rarely seen in non-territorial males. The AVP-V₁ receptor antagonist had opposite effects. It
decreased courtship and territorial defense, making these males act more like non-territorial males. These experiments demonstrated that manipulations of the AVT system shifted males within a single phenotype from the non-territorial social status to the territorial social status and vice-versa.

Additionally, we examined the role of social and gonadal inputs on the AVT system in the preoptic area (POA) of the hypothalamus. In one experiment, we found that AVT mRNA abundance is higher in sex-changing females who attain social dominance and display dominant male behavior than in subordinate females, regardless of whether the dominant females were intact or ovariectomized. However, AVT-ir soma size in the gigantocellular POA (but not magnocellular or parvocellular POA) increased only when females were both displaying dominant male behavior and had developed testes. In a second experiment, castration of dominant terminal phase males had no effect on AVT mRNA abundance or any behavior we measured but did increase gPOA AVT-ir soma size compared with sham-operated controls. In the third experiment, implants of 11-ketotestosterone (11KT), the potent teleost androgen, in socially subordinate, ovariectomized females had no effect on either AVT mRNA abundance or AVT-ir soma size compared with controls. These results demonstrate that AVT neural phenotype in the bluehead wrasse can be strongly influenced by social status and these social influences can be manifested independent of gonads.

Furthermore, we examined the roles of AVT and 11KT in mediating sexual and aggressive behaviors typical of dominant males. We demonstrated that AVT appears necessary for the assumption of dominant territorial status in males and females, but is not sufficient for the display of male behavior in females or initial phase males. Finally,
treating females with 11KT did not alter responsiveness to AVT, but did induce male coloration and courtship behavior that was not observed in oil-treated females. Together these results indicate that the ability of AVT to induce male-typical behavior differs among sexual phenotypes and that this differential responsiveness appears to be mediated by social context and not directly by exposure to 11KT. Since 11KT can induce courtship behavior in females that is not affected by AVT, there may be different hormonal mechanisms mediating courtship behavior under different social contexts.
HORMONAL MECHANISMS REGULATING ALTERNATE PHENOTYPES

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A dissertation submitted to the Graduate Faculty of North Carolina State University In partial fulfillment of the Requirements for the Degree of Doctor of Philosophy

Zoology

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DEDICATION

To my parents Jim and Anne Semsar. Thank you for all the struggles you endured to give Chris and Cary and me the opportunities we needed to fly, and the acceptance and love and encouragement you gave us as we did.
Katharine Semsar was born in 1974 in New York City, New York. Shortly after her first birthday, her family moved to Baraboo, Wisconsin, where she lived through high school, grateful for Devil’s Lake State Park as her backyard. After graduating from Baraboo Senior High School in 1992, Katharine began her undergraduate degree at University of California, Santa Cruz in hopes of becoming a marine biologist. In 1995, she transferred to University of Wisconsin, Madison. While in Madison, Katharine worked with Dr. Catherine Marler, eventually publishing work initiated as her senior thesis. In 1996, she graduated with an Honors B.Sc. in Zoology and stayed one year in Madison during which time she discovered her passion for teaching while working as a teaching assistant for UW Madison’s Introductory Biology course.

Following her year teaching, Katharine took a year to travel worldwide. Her travels included two months at Cao Hai Nature Reserve in Guiyang Province, China where she worked with the International Crane Foundation (Baraboo, WI) and the Cao Hai staff to develop environmental education programs in the elementary schools. She also spent two months traveling in Thailand and another month in Europe, paying for her travels by doing temporary work at Circus World Museum, Baraboo, WI, between trips.

In 1998, Katharine came to NC State under the mentorship of Dr. John Godwin to pursue a doctoral degree in behavioral biology. After completion of her degree she is pursuing post doctoral work with Dr. Tim Clutton-Brock at Cambridge University where she plans to study hormone-behavior relationships in wild populations of meerkats in the Kalahari Desert of South Africa.
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CHAPTER I

INTRODUCTION

Both environmental and endogenous inputs influence the display of sexual and aggressive behavior. Many ontogenetic and predictable environmental cues, such as photoperiod, provide important information for the appropriate display of sexual and aggressive behavior. However, for animals living in complex social environments, social stimuli may be better predictors of when these behaviors should be displayed. Furthermore, when social environments are unpredictable, individuals must be able to adapt to these changes by rapidly modifying their behavior. These behavioral responses are likely to involve actions of neuropeptides in the brain. Therefore, an understanding of rapid-acting neuropeptide pathways is therefore crucial to an understanding of how animals adapt to their social environment.

Arginine vasotocin (AVT) and vasopressin (AVP) are members of a highly conserved neuropeptide family and are important both for osmoregulation and for the display of sexual and aggressive behavior. These nonapeptides show both physical and functional conservation through the evolution of vertebrates. AVP and AVT differ in only one amino acid. Likewise, they modify the display of sexual behavior and aggression in species of all vertebrate classes. In addition, cells producing AVT/AVP are found in areas of the brain, such as the hypothalamus and amygdala, that are primary integrative areas for social stimuli and male-typical behavior. Sexual dimorphisms in these brain regions and castration/replacement studies suggest that AVT/AVP interacts with androgens. Unlike androgens which have permissive effects on behavior, AVT
seems to play a more causal role modifying behavior. Although a growing number of studies address these effects, much less is known about how AVT manipulations affect behavior under either variable social contexts or natural conditions. Additionally to date, very few studies address both behavioral effects of AVP/AVT and their potential adaptive value. Understanding how these peptides affect behavior under variable social conditions and how they interact with other hormones will help us understand the underlying mechanisms by which animals, including humans, adapt their behavior in response to their social environment (for reviews see Goodson and Bass 2001, DeVries and Simerly 2002).

The bluehead wrasse (*Thalassoma bifasciatum*) is an excellent model system for approaching these questions for many reasons. First, abundant on Caribbean coral reefs, the bluehead wrasse has a complex and well-studied social system that is also easily manipulated and studied under natural conditions. Second, life-history characteristics such as socially-controlled female-to-male sex change and distinct alternate male phenotypes are "natural experiments" that provide pronounced behavioral variation within and among individuals. Furthermore, bluehead wrasses can undergo behavioral sex change even after the surgical removal of their gonads (Godwin et al. 1996). This decoupling of the brain from gonadal influences can be exploited to more directly examine other influences, such as social interactions, on the neural substrates of reproductive behavior. The exceptional combination of a complex social structure, unusual but experimentally valuable reproductive biology, and ease of observation and manipulation in nature makes the bluehead wrasse a powerful model in which to explore these mechanisms of behavioral adaptation to social complexity.
Both AVT and 11-ketotestosterone (11KT), the dominant androgen in male teleosts, increase with a rise in social status from negligible levels in females to intermediate levels in IP and non-territorial TP males to high levels in territorial TP males (Grammer, 1998; Godwin et al. 2000). It is not known whether the hormonal changes are causes or consequences of social status, but these patterns provide the necessary background to begin meaningful and hypothesis-driven manipulations of the system.

In my dissertation I have sought to further understand the role of AVT relative to social context, gonadal input, and androgen influences in the mediation of the development and maintenance of male behavior. To address this goal, I designed experiments following one of two basic designs: manipulating AVT levels under variable social contexts and measuring subsequent behavior, or manipulating combinations of social, gonadal, and androgen levels and measuring both behavioral and AVT responses. Along these lines, Chapter II describes how manipulations of the AVT system can shift social status among T-TP and NT-TP males. Chapter III describes how social, gonadal, and androgen influences interact to influence the AVT phenotype, specifically AVT mRNA abundances and AVT-ir cell size. Chapter IV describes the relative influences of AVT and 11KT to influence male behavior across varying social contexts and sexual phenotypes. Chapter V summarizes what we have learned about the role of AVT in the mediation of responses to environmental signals. This summary is placed in the larger context of how understanding the predictive value of environmental cues to the timing of optimal reproductive opportunities can aid in the elucidation of hormonal mechanisms mediating phenotypic changes associated with variable reproductive states.
Since Chapters II, III, and IV have been written for separate publication and have either already been printed or have been accepted for publication, they have been presented as reprints or manuscripts in the style of the journals where they are or will be published. Therefore, there has necessarily been some overlap of information among chapters and stylistic differences as required by the publication sources.
CHAPTER II
MANIPULATIONS OF THE AVT SYSTEM SHIFT SOCIAL STATUS IN
THE BLUEHEAD WRASSE

This chapter has been previously published as:


CHAPTER III
SOCIAL INFLUENCES ON THE AVT SYSTEM ARE
INDEPENDENT OF GONADS IN THE BLUEHEAD WRASSE

This chapter has been previously published as:

Social Influences on the Arginine Vasotocin System Are Independent of Gonads in a Sex-Changing Fish

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Many neuropeptide systems subserving sex-typical behavior are dependent on sex steroids for both their organization early in life and activation during maturity. The arginine vasopressin/vasotocin (AVP/AVT) system is strongly androgen dependent in many species and critically mediates responses to sociosexual stimuli. The bluehead wrasse is a teleost fish that exhibits a female-to-male sex change in response to social cues, and neither the development nor the maintenance of male-typical behavior depends on the presence of gonads. To examine social and gonadal inputs on the AVP/AVT system in the preoptic area (POA) of the hypothalamus, we conducted three field experiments. In the first experiment, we found that AVT mRNA abundance is higher in sex-changing females that attain social dominance and display dominant male behavior than in subordinate females, regardless of whether the dominant females were intact or ovariectomized. However, AVT-immunoreactive (IR) soma size in the gigantocellular POA (gPOA), but not in the magnocellular or parvocellular POA, increased only when females were displaying both dominant male behavior and had developed testes. In the second experiment, castration of dominant terminal-phase males had no effect on AVT mRNA abundance or any behavior we measured but did increase gPOA AVT-IR soma size compared with sham-operated controls. In the third experiment, 11-ketotestosterone implants in socially subordinate, ovariectomized females had no effect on either AVT mRNA abundance or AVT-IR soma size compared with controls. These results demonstrate that the AVT neural phenotype in the bluehead wrasse can be strongly influenced by social status, and that these social influences can be manifested independent of gonads.

Key words: arginine vasotocin; neuropeptide; androgens; gonadal steroids; social behavior; reproductive behavior; 11-ketotestosterone; preoptic area; field study

Introduction

Gonadal sex steroids typically play key roles in the organization of sex-typical behavior in many vertebrates. Steroids can influence behavior by directing neural growth, connectivity, and neuro-hormone systems in the brain that are important in sex-typical behavior (Arnold and Breedlove, 1985; Sachs and Meisel, 1994; De Vries and Simerly, 2002). An important influence on male-typical behavior is the interaction between androgens and the arginine vasopressin/vasotocin (AVP/AVT) system, a key neuropeptide mediator of social behavior. During both development and adulthood in numerous species, castration and androgen inhibitors decrease forebrain AVP/AVT immunoreactivity and mRNA levels, AVP/AVT receptor expression, and behaviors mediated by these peptides, whereas androgen replacement reverses these effects (for review, see Goodson and Bass, 2001; De Vries and Simerly, 2002).

However, the organization of sex-typical behavior in species with environmental sex determination may be mediated by factors other than gonadal steroids. For example, temperature directly affects both brain morphology and function in the leopard gecko, Eublepharis macularius, a species with temperature-mediated sex determination (Crews et al., 1996). In the bluehead wrasse (Thalassoma bifasciatum), a sex-changing teleost fish, the dominant male sexual phenotype develops when large females become socially dominant (Warner, 1988; Warner and Swearer, 1991). Importantly, behavioral sex change can be very rapid, and ovariectomy does not prevent the development of male behavior in socially dominant females (Godwin et al., 1996). Therefore, the bluehead wrasse is a useful model for studying the effects of social environment on the function of neural systems subserving sex-typical social behavior.

Bluehead wrasse populations include females and initial-phase (IP) and terminal-phase (TP) males. Territorial TP males actively defend spawning sites where they court and spawn with females (Warner, 1988). Removing TP males from small reefs induces behavioral and morphological sex change in the largest females (Warner and Swearer, 1991; Godwin et al., 1996). Several days after testes begin developing, 11-ketotestosterone (11KT) levels increase (our unpublished data). Analogous to dihydrotestosterone in tetrapods, 11KT mediates male secondary sexual character development in fish (for review, see Borg, 1994; Grober and Bass, 2002). However, unlike gonadal change, the onset of male-typical behavior in females can occur within minutes to hours (Warner and Swearer, 1991) and is correlated with increases in hypothalamic AVT mRNA within 2–3 d [the earliest time point sampled by Godwin et al. (2000)]. Furthermore, AVT is necessary for the display of male behavior during early sex change (our unpublished data) and is both necessary for and can
induce the display of territorial behavior in intact TP males (Semsar et al., 2001). Because the acquisition of dominant male behavior in bluehead wrasses does not depend on testes but does depend on AVT, we tested the interactions of social status, gonadectomy, and androgen replacement on the AVT phenotype of free-living bluehead wrasses. Specifically, we hypothesized that: (1) AVT phenotype would differ in sex-changing females attaining social dominance compared with subordinate females, regardless of gonadal status, (2) AVT phenotype and territorial behavior in TP males would be unaffected by castration, and (3) AVT phenotype in socially subordinate, ovariectomized females would be unaffected by 11KT administration.

Materials and Methods

Study sites. The field portions of the experiments examining the effects of ovariectomy and social status on the development of the AVT phenotype during sex change were conducted from May through July of 2000 and 2001 on small patch reefs with one to four spawning sites in Teague Bay, St. Croix, and the U.S. Virgin Islands (reefs 4, 9a, 9b, 10, 12, 14, 16b, 18, and 21) [for description of reefs, see Gladfelter and Gladfelter (1987)]. Experiments examining the role of castration on the maintenance of the AVT phenotype during TP male dominance were conducted from May through August 2000 and 2001 on large patch reefs with five to nine spawning sites in Teague Bay (reefs 6, 11, 13, and 22). The experiment examining the role of 11KT on the development of the AVT phenotype under social inhibition was conducted in April and May of 2002 on coral patch reefs in the lagoon of Glover’s Reef Atoll, Belize [for description of reefs, see Stoddart (1962)].

Field experiment 1: social and gonadal influences during sex change. This experiment was designed to test the interactions of social and gonadal influences on the AVT system in sex-changing fish using experimental groups that provide clear behavioral contrasts [previously characterized by Godwin et al. (1996)]. In 2000, we captured all IP individuals (both females and males) that were >45 mm standard length (SL) from small patch reefs and permanently relocated IP males to different reefs. We either ovariectomized or sham-operated the largest five to eight females (range, 50.1–74.9 mm SL; mean, 61.6 mm) as described by Godwin et al. (1996), balancing treatment across body size. We tagged the experimental females for individual identification using beaded floy tags (Warner and Schultz, 1992; Semsar et al., 2001) and returned them to their home reef 2–3 d after surgery. After allowing 2 d of recovery from surgery, we induced sex change in the largest females on the reef by relocating all TP males to distant reefs. During the following week, we periodically checked the reefs for immigration of TP males from neighboring reefs and removed them, if found. If TP males were found repeatedly on a reef, we did not include those females in the study.

Seven days after TP removal, we observed experimental females on the reef during the spawning period (midday) and categorized each remaining experimental female as either dominant or subordinate (behavioral data not shown). As in a previous study (Godwin et al., 1996), dominant individuals were those displaying behavior that was typical of territorial TP males, including aggressively defending and actively courting on a spawning site, whereas subordinate individuals were not observed displaying any of these behaviors. Hence, on each reef, we had four classifications of individuals: dominant-ovariectomized (DO), dominant-sham (DS), subordinate-ovariectomized (SO), and subordinate-sham (SS). Immediately after the spawning period (2:30 P.M. to 4:00 P.M.), we captured experimental fish, transferred them to a small boat, and killed them with an overdose of the anesthetic tricaine methyl sulfonate. We then noted whether individuals had any permanent blue coloration, a TP male characteristic that develops reliably in DS females and not in DO females (Godwin et al., 1996). Color develops under the influence of 11KT, as demonstrated by the field hormone treatments in experiment 3 and pilot experiments on captive ovariectomized females given 11KT (our unpublished data). To measure circulating levels of 11KT, we immediately drew blood samples from the caudal vein, placed the samples on ice, and centrifuged these samples at 3000 × g 2–6 hr later to collect plasma. Plasma was stored at −20 to −80°C until analysis. To examine the AVT system, we removed the brains immediately after killing, immersion-fixed them in 4% paraformaldehyde for 24 hr, and then stored them in 0.1 M PBS at 4°C until cryosectioning. Finally, we dissected the bodies to verify the success of ovariectomies and determine the gonad identity (testes or ovary) of sham-operated individuals.

In 2001, we repeated the experiment with one design change. This design change was intended to determine whether the AVT system in subordinate females differed depending on whether they were subordinate to established TP males or to newly dominant and sex-changing individuals. Therefore, after females underwent surgery and were returned to their home reef, we manipulated the reefs to create either dominant or subordinate females. On reefs designated for dominant females (n = 2), we repeated the methods used in 2000 and relocated all TP males from the reef 2 d after surgery. From these reefs, we only collected females who became dominant on spawning sites. On reefs designated for subordinate females (n = 4), we controlled for disturbance associated with fish capture by catching TP males 2 d after surgery, holding them for 10 min in a net, and releasing them back on the focal reef. All females collected from these reefs remained subordinate to the TP males on the reefs. Consequently, in 2001, all subordinate females were subordinate to TP males rather than to sex-changers as in 2000. A second consequence of this altered design is that we were able to collect subordinate fish that were larger and therefore comparable in size with dominant fish collected in 2000. In addition, because of field complications, females in 2001 were sampled 10 d (rather than 7 d) after TP males were removed and/or manipulated.

Field experiment 2: castration effects on dominant TP males. This experiment was designed to test the effects of castration on social status, territorial behavior, and AVT phenotype of territorial TP males. In 2000, we caught all TP males from large patch reefs and tagged them for individual identification using beaded floy tags (Floy Tag, Seattle, WA) (Warner and Schultz, 1992). Two to 3 d after tagging (to allow for assessment of whether TP males were territorial), we caught the territorial males in the morning (leaving the nonterritorial TP males on the reef throughout the entire duration of the experiment), either castrated them or performed a sham operation [8:00 A.M. to 11:00 A.M., using the same general surgical methods as outlined for ovariectomies by Godwin et al. (1996)], and then returned them to the reef before the spawning period. The number of castrated and sham-operated males was balanced across body size on each reef (range, 70.7–94.1 mm SL; mean, 85.5 mm). We observed focal males 21–22 d after surgery in the alternating morning and either castrated or sham-operated them for 2–3 d after surgery, recording the frequency and duration of both courtships and aggressions and the frequency of inspections and spawns [for a detailed description of these behaviors, see Semsar et al. (2001)]. These data were used to verify territorial status in males. After the spawning period, we sampled the focal males as described above for females.

In 2001, we followed the same general experimental methods. However, because we had seen no clear behavior differences between treatments in 2000, we included additional behavioral observations and experiments to more fully test for differences between castrated and sham-operated males. First, 1–3 d after tagging, we observed males to obtain baseline behavioral measures for each on the same suite of behaviors noted above. Within 4 d of observing males, all territorial TP males on a reef were caught in the morning and either castrated or sham-operated and then held until the late afternoon (~5:00 P.M.) and returned to the reef. Again, we balanced treatments across body size (range, 84.1–93.2 mm SL; mean, 88.9 mm) but with a bias toward sham-operated males because we had recovered fewer sham-operated males in 2000. Two to 3 d after surgeries, we observed the focal males for the standard suite of territorial male behaviors. We exposed males to standardized behavioral tests designed to detect differences between treatments in responses to predators and TP male intruders 14–20 d after surgery. The predator test was performed first (14–16 d after surgery), and the intruder test was performed 2–4 d after the predator test (16–20 d after surgery).

To test responses to predators, a lizardfish (preserved and weighted) was introduced into the focal male’s territory. The lizardfish, Synodus...
intermedius, is a sit-and-wait predator common on Teague Bay reefs, and preserved fish act as realistic model predators to blueheads (Warner and Dill, 2000). First, we recorded normal territorial behavior for 10 min. We then presented the model to the focal male by waiting until the male was briefly away from his territory and placing the model within 0.5 m of the focal male’s spawning site. When the focal male reentered its territory, we recorded the latency of the male to resume courting on its spawning site, the duration of time it spent on its territory, the frequency of bobs (a behavior commonly displayed in the presence of predators) (Warner and Dill, 2000), and the standard suite of territorial behaviors for two 10 min time budgets. We then removed the lizardfish model and recorded the latency of the focal male to resume courtship on its spawning site.

To test responses to intruder TP males, we presented focal males with a live TP male intruder housed in a Plexiglas tube (8.2 \times 1100 \times 3.0 \text{ cm}) screened ends allowed water to flow through, a weight was strung with monofilament to the bottom, and a float was strung with monofilament (~0.5 m above the tube). The same intruder was used in all trials and was smaller than each of the focal males (82.4 mm SL). During the spawning period, we placed the model within 0.5 m of the spawning site while the focal male was briefly away from its territory. Because the area defended by individual territorial males extends a minimum of several meters along the edge of reefs, these intruders were well within the focal male’s territorial boundaries. When the focal male reentered its territory, we recorded the latency to first approach or contact with the tube and the frequency of approaches and contacts with the tube for 5 min. Approaches were defined as the focal male approaching within one body length of the tube but not touching the tube. Contacts were defined as approaches ending in physical contact with the tube. Each approach or contact ended if the focal male turned away from the tube. After 5 min, the intruder was removed from the site.

Finally, 21–23 d after surgery, we again observed the focal males in alternating periods of 10 min throughout the entire spawning period, recording all territorial and courtship behaviors during the focal sample. At the end of the spawning period, focal males were caught and sampled as described above.

Field experiment 3: 11KT effects on the AVT system in ovarietomized females. This experiment was designed to test the effect of 11KT, the teleost androgen that induces secondary sexual characteristics (for review, see Borg, 1994), on the AVT system under conditions that socially inhibit sex change. We caught, tagged, and ovarietomized the largest four to eight females as described above (range, 57–70.1 mm SL; mean, 64.8 mm) from patch reefs with two to 10 TF males present. Each female received an 8 mm Silastic implant (~20 μl; Silastic tubing, 1.47 mm inner diameter, 1.96 mm outer diameter; Dow Corning, Midland, MI) of either castor oil or castor oil plus 11KT (5 μg/μl; Steraloids, Newport, RI). The dose was chosen on the basis of effectiveness of implants used in goldfish (Stacey and Kobayashi, 1996) and pilot studies in bluehead wrasse showing that ovarietomized female implants with this dose of 11KT showed full color development within 2 weeks, similar to natural sex changers, which take ~3 weeks (2 weeks after full testes development). In addition, although there are no published data on 11KT levels in the bluehead wrasse, the levels we induced here were comparable with those from intact TP males sampled from Glover’s Reef in 1997 (mean, 1344 ± 485 pg/ml; range, 435–2900 pg/ml; K. Sensar, J. Godwin, M. Grober, unpublished data), and within the approximate range for TP males in other sex-changing fish (Cardwell and Liley, 1991; Hourigan et al., 1991). The implanted females were released the same day to their home reef, and no additional social manipulations were performed. Ten to 13 d after the surgery, we tested whether these females would respond to AVT by giving each a series of two intraperitoneal injections of saline and AVT (1 μg/gm body weight) on subsequent days [methods followed those of Sensar et al. (2001)]. The behavioral portion of this experiment is part of a larger and more comprehensive set of experiments addressing behavioral effects of exogenous AVT treatment across phenotypes, which is beyond the scope of this study. Fourteen to 16 d after surgery (and at least 2 d after hormone injections), we recaptured the females and photographed them to document color pattern differences and then collected blood and brain samples as described above.

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

11-ketotestosterone assays. Levels of 11KT in unextracted plasma samples were assessed using an 11KT enzyme-linked immunosassay according to the directions of the manufacturer (ELISA kit 582751; Cayman Chemicals, Ann Arbor, MI). Because of small plasma volumes, particularly for smaller females and sex changers, only one 20 μl aliquot of each sample was assayed, and sample sizes were small in some groups. Samples were incubated for 2 hr, and color development was read using a Bio-Tek Kinetic Reader (Bio-Tek Instruments, Winooski, VT). Unextracted plasma 11KT levels were then corrected using data from additional assays comparing unextracted and extracted plasma pool samples. To do this, we extracted a portion of the original plasma pool (derived from females, TP males, and TP males twice with diethyl ether) and ran these extracted samples and unextracted samples (original plasma pool) in parallel. In addition, a portion of these samples was spiked with a known amount of 11KT. Although these comparisons did not assess the correction for the three phenotypes individually, measurements of the extracted versus unextracted pools and spiked aliquots from the pool were consistent in indicating that 11KT levels were underestimated by 43% in unextracted samples; therefore, unextracted values were divided by 0.57.

AVT mRNA in situ hybridization. AVT mRNA relative abundances in the preoptic area (POA) of the hypothalamus were analyzed using in situ hybridization (ISH). The methods used are described briefly below and have been described in detail previously by Godwin et al. (2000). All brains were cryosectioned into six adjacent series of 20 μm sections, and one series from each individual was used for ISH. For TP males from 2000, the ISH procedure used the same 33 mer degenerate antisense oligonucleotide probe for teleost AVT labeled with 32P that was used by Godwin et al. (2000). For all females (2000 and 2001), sex-changers, and TP males from 2001, a specific antisense probe (5’-AACAAATGAG-GTCTTGCAAGGGGCTCCTCCCTT-3’) based on the T. bifasciatum AVT sequence was used (GenBank accession number AY167033). Using a sense strand probe complementary to this sequence generates no signal, and previous RNase treatment eliminates signal (our unpublished data). Additionally, the pattern of labeling generated by both the homologous and degenerate oligonucleotide probes is identical to that generated with a homologous cRNA probe derived from a cloned T. bifasciatum AVT cDNA (our unpublished data).
Regions of consistent size placed over groups of cells in the POA were measured for average silver grain counts using Brain software (version 3.0; Drexel University Computer Imaging and Vision Center, Philadelphia, PA) on a Macintosh Power personal computer. Background was controlled by subtracting the measurement from an identically sized region located outside of the POA on the same section from the measurement of cells in the POA.

Importantly, independent measures of hybridization signal from phosphor imaging screens for both TP males and sex changers showed that 88 and 91%, respectively, of total hybridization signal was accounted for by sections containing only giantcellular and magnocellular neurons. In addition, total silver grain counts are significantly correlated with total hybridization signal \( R^2 = 0.73; p < 0.0001 \). Except where noted, we present data on hybridization signal from phosphor imaging screens, because their large dynamic range prevents ceiling effects that can occur with silver grain counts.

For the ovariectomy experiment that examined the development of the AVT system, all slides from 2000 and 2001 were run in the same ISH procedure; therefore, comparisons between years could be made. For the castration experiment that examined the maintenance of the AVT system in TP males, males from each experimental year were run in a separate assay. Because hybridization signals depend on several assay-specific factors in ISH (e.g., label-specific activity, exposure time), we cannot distinguish whether differences between assays were attributable to assay or year differences in the castration experiment.

**AVT immunocytochemistry.** To examine AVT-immunoreactive (IR) cell size in the POA, a second series of slides from the original six series cut from each individual was analyzed using immunocytochemistry (ICC). Therefore, the POA from each individual in these experiments was analyzed by both ISH and ICC. Our ICC protocol followed that put forth by Foran and Bass (1998), using a primary antibody to rat AVP (DiaSorin, Stillwater, MN), peroxidase ABC kits (Vector Laboratories, Burlingame, CA) for secondary and tertiary antibodies, and DAB intensified with 0.3% NiCl₂. We estimated the number of AVT-IR cells and the average cross-sectional area of AVT-IR soma size in the POA using Brain software (version 3.0). Each cell was assigned to one of three POA nuclei (POA) based on cell location, fiber extension, and descriptions of these areas as described by Braford and Northcutt (1983). These regions are the parvocellular POA (pPOA), located in the anteroventral POA often without obvious fibers (Fig. 1C); the magnocellular POA (mPOA), located in the medial POA with obvious fiber extensions toward the pituitary (Fig. 1B); and the gigantocellular POA (gPOA), reported only in teleosts and located posterior and dorsal to the mPOA with fiber extensions that are not obviously directed toward the pituitary (Fig. 1A). We tested the appropriateness of using these criteria in assigning AVT-IR neurons to different POA regions and found that average AVT-IR soma sizes were significantly different among them (one-way ANOVA; \( F = 51.63; p < 0.001 \); all Tukey honestly significant difference comparisons; \( p < 0.05 \)) (Fig. 1D). In the gPOA and mPOA, we measured only AVT-IR cells showing at least one neurite. In the pPOA, we do not see neurites on cells in coronal sections. However, these cells are small (diameter, \( \sim 5 \mu m \)), greatly increasing the likelihood that entire cells are represented within single sections (thickness, 20 \( \mu m \)).

**Statistical analyses.** ELISA data on 11KT plasma levels were analyzed with one-way ANOVA and Tukey–Kramer post hoc tests, where appropriate. Because of an inability to obtain plasma samples from each individual, only the subset of animals in which plasma samples were obtained is represented. Those individuals with levels of 11KT below detection limits were included with values assigned as the minimum detection limits for the assay.

There were no significant differences in body size among treatments in any of the experiments presented here \( (p > 0.3 \) for all experiments). However, both measures of AVT expression, hybridization signal, and AVT-IR soma size are correlated with body size. Therefore, all subsequent analyses use data corrected for body size by dividing the respective measure by the standard length of the animal.

Total hybridization signal for AVT mRNA was analyzed using ANOVA and post hoc orthogonal contrasts where appropriate. In experiment 1, in which all individuals were in the same assay, a three-way ANOVA compared the effects of year, dominance, and presence of gonads. In experiment 2, in which each year was run in a separate assay, a two-way ANOVA compared the effects of assay and treatment. In experiment 3, a Student’s \( t \) test assessed the effect of treatment.

For analyses of AVT-IR POA soma sizes, no differences in cell size were found for animals from different experimental years in any experiment (including between females subordinate to TP males and those subordinate to sex changers; \( p > 0.1 \) for all experiments), and data were therefore combined. Cell sizes from the gPOA, mPOA, and pPOA were analyzed separately. Sample sizes for the three POA regions do not always match because of the occasional loss of sections within a single area during ICC. The appropriate effects were again compared using ANOVA and post hoc orthogonal contrasts. In addition, potential correlations between average gPOA AVT-IR soma sizes and gPOA grains per cell were tested with Pearson’s correlation.

Finally, behavioral experiments were analyzed using paired sample tests to compare frequencies and duration of behaviors and Fisher’s exact tests to compare the occurrence of courtship and predator responses (see Table 2).

**Results**

**Effects of gonad removal and hormone implants on 11KT levels and color patterns**

Gonad removal and hormone replacements effectively generated differences in 11KT levels among treatment groups (Table 1). Despite small sample sizes in some groups in experiment 1, females undergoing sex change with gonads (DS) had 11KT levels comparable with sham–operated TP males and higher levels than the other female groups (DO, SO, or SS; \( p = 0.02 \)). Importantly, all DS females showed the development of permanent blue coloration on the head, whereas none of the other treatment groups did, including the dominant ovariectomized females (DO). This coloration reliably develops under the influence of 11KT as mentioned above in Materials and Methods. In experiment 2, castrated males had significantly lower 11KT levels than sham-operated males \( (p = 0.02) \). In addition, all sham-operated males had blue skin healing around the surgical incision, whereas all castrated males had only white skin healing around the incision. In experiment 3, although once again we were able to collect only small numbers of plasma samples, the two samples from oil
treated females had nondetectable levels of 11KT, whereas 11KT-treated females had elevated 11KT levels.

**Experiment 1: social and gonadal influences during sex change**

**AVT mRNA abundance**

There was no effect of experimental year on AVT mRNA signal (p > 0.5), indicating that females subordinate to sex changers and those subordinate to TP males did not differ. Therefore, all SO and SS females from both years were grouped in subsequent analyses. The acquisition of dominance on a spawning site was associated with a nearly twofold increase in AVT mRNA signal (p = 0.001) (Fig. 2A), whereas the presence of gonads had no effect (p = 0.82) (Fig. 2A). This difference in AVT mRNA abundance between dominant and subordinate females appears to be attributable to differences in the mPOA for two reasons. First, sections containing parvocellular neurons only accounted for 9% of the hybridization signal as measured by phosphor imaging. Second, there was no significant effect of dominance on silver grain densities in the gPOA (p = 0.83) or pPOA (p = 0.11), whereas there was a significant difference in the mPOA (p = 0.035; data not shown).

**AVT-IR POA soma size and number**

The average AVT-IR soma size of the mPOA and pPOA did not vary across treatments. However, the average AVT-IR soma size in the gPOA of intact, sex-changing (DS) individuals was significantly larger than those in the three other treatment groups (orthogonal contrast, DS > DO, SO, SS; p = 0.04) (Fig. 2B). However, increased gPOA AVT-IR soma size in DS individuals did not correlate with increased AVT mRNA signal in those neurons as measured by silver grains per cell (p > 0.1). There was no effect of treatment on AVT-IR cell number in any of the three nuclei (p > 0.1 for all experiments; data not shown).

**Experiment 2: castration effects on dominant TP males**

**AVT mRNA abundance**

Castration did not affect AVT mRNA signal (p = 0.71) (Fig. 3A). Although there was an effect of year and/or assay on hybridization signal (p < 0.001), this most likely represents assay differences typical of ISH rather than variation in message abundance between years (samples were run in two separate assays).

**AVT-IR POA soma size and number**

Average AVT-IR soma size did not differ between sham and castrated males in either the mPOA (p = 0.13) (Fig. 3A) or pPOA (p = 0.53) (Fig. 3A). Average AVT-IR soma size in the gPOA was significantly larger in castrated males than in sham-operated males (p = 0.01) (Fig. 3A). However, as with the DS group in the sex change experiment described above, average gPOA AVT-IR soma size did not correlate with AVT mRNA signal measured as grains per cell ($R^2 = 0.17$; p > 0.1). There was no effect of treatment on AVT-IR cell number in any of the three nuclei (p > 0.1 for all experiments; data not shown).

**Behavior**

All TP males found on the reefs at the end of the experiments were territorial. No measure of male behavior in 2001 showed significant differences when compared either before and after surgery or between castrated and sham-operated males (Table 2).

**Experiment 3: 11KT effects on the APT/AVT system in ovariolectomized females**

All ovariolectomized females implanted with 11KT showed either full development of TP male coloration or were in the late stages of developing this coloration (blue head, green tail, near complete development of the black–white–black barring behind the pectoral fin insertion) during recapture. No females who received a control implant showed evidence of color change.

**AVT mRNA abundance**

In these socially subordinate and ovariolectomized females, AVT mRNA expression did not vary with 11KT treatment (p > 0.1) (Fig. 4A).
In socially subordinate and ovariectomized females, average AVT-IR soma sizes in all three POA regions did not vary with 11KT treatment ($p/H_{11022} 0.1$) (Fig. 4B). There was no effect of treatment on AVT-IR cell number in any of the three nuclei ($p/H_{11022} 0.1$ for all experiments; data not shown).

**Discussion**

Gonadal sex steroids play critical roles in the organization and maintenance of sexually dimorphic brain systems in a variety of vertebrate species. These hormones are particularly important in the organization and activation of the AVP/AVT system and are an important mediator of male social behavior in well characterized rodent, amphibian, and avian models (for review, see Goodson and Bass, 2001; De Vries and Simerly, 2002). Although the AVP/AVT system can also respond to social influences (Delville et al., 1998), it is unclear to what degree these responses to social input are mediated through the hypothalamo-pituitary-gonadal (HPG) axis or other signaling systems in the brain. We show here that in bluehead wrasses, the AVT system can develop and be maintained independent of gonadal function. Therefore, bluehead wrasses provide an unusual and useful model to study how social environments can affect neuropeptide function through nongonadal pathways.

**AVT mRNA abundances are predicted by behavior**

Many fish, especially sex-changing species, rely on social cues rather than the ontogenetic maturation of gonads to initiate and orchestrate the development of the dominant phenotype (Warner, 1988). Studies to date have focused primarily on how social cues modulate the HPG axis to affect both morphological characters and neural substrates of behavioral characters (Grober et al., 1991; Francis et al., 1993; Fernald, 2002). However, in bluehead wrasses, the development of dominant male behavior in natural sex-changers can precede the change of ovaries to testes (Warner and Swearer, 1991) and occurs even if females are ovariectomized (Godwin et al., 1996). We show here that this gonad-independent switch from female to male behavior is accompanied by a gonad-independent increase in AVT mRNA abundance in the mPOA. The mPOA and gPOA of fish are considered homologous to the paraventricular nucleus of tetrapods (Braford and Northcutt, 1983). Although the mPOA does project to the pituitary, both nuclei also project to at least the telencephalon and the thalamus in trout (Saito et al., 2002). Therefore, it is likely that AVT changes in these regions could directly alter neural function in other brain areas.

All three experiments show that during the development and

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**Table 2. Measures and $p$ values of all behavioral tests from the TP male castration experiment conducted in 2001**

<table>
<thead>
<tr>
<th>Behavior experiment</th>
<th>Sample size</th>
<th>Measure</th>
<th>Test</th>
<th>$p$ value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Pre–Post Territorial TP behavior</td>
<td><em>(n = 9)</em></td>
<td>Courtship (f, dur)</td>
<td>Paired t test</td>
<td>0.45, 0.19</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Aggression (f, dur)</td>
<td>Paired t test</td>
<td>0.21, 0.22</td>
</tr>
<tr>
<td></td>
<td></td>
<td>f of inspections</td>
<td>Paired t test</td>
<td>0.39</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Spawning</td>
<td>Paired t test</td>
<td>0.71</td>
</tr>
<tr>
<td>Response to predator</td>
<td>S = 9</td>
<td>Presence of courtship</td>
<td>Fisher exact</td>
<td>1</td>
</tr>
<tr>
<td></td>
<td>C = 4</td>
<td>Presence of bobbing</td>
<td>Fisher exact</td>
<td>1</td>
</tr>
<tr>
<td>Response to TP Intruder</td>
<td>S = 6</td>
<td>f of approaches</td>
<td>Wilcoxon</td>
<td>0.91</td>
</tr>
<tr>
<td></td>
<td>C = 4</td>
<td>f of contacts</td>
<td>Wilcoxon</td>
<td>0.51</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Latency to approach</td>
<td>Wilcoxon</td>
<td>0.59</td>
</tr>
</tbody>
</table>

$^1$ Frequency; dur, duration; C, castrate male; S, sham control.

**AVT-IR POA soma sizes**

In socially subordinate and ovariectomized females, average AVT-IR soma sizes in all three POA regions did not vary with 11KT treatment ($p > 0.1$) (Fig. 4B). There was no effect of treatment on AVT-IR cell number in any of the three nuclei ($p > 0.1$ for all experiments; data not shown).
maintenance of the dominant male phenotype, social cues and changes in social status can alter AVT mRNA abundance through pathways independent of gonadal status and circulating 11KT. First, among dominant sex changers, individuals with functioning gonads (DS) and correspondingly higher 11KT levels show no additional increase in AVT mRNA abundances over ovarietomized individuals (DO), although both groups have significantly higher AVT mRNA abundances than subordinate groups. Second, castrated TP males show AVT mRNA abundances similar to those of sham-operated males despite significantly reduced 11KT levels (experiment 3) exhibit low AVT mRNA abundances when kept under natural socially subordinate conditions.

Although neither the presence of testes nor 11KT predicted AVT mRNA abundances in these experiments, AVT mRNA levels were predicted by the presence of dominant male behavior. Consistent with the findings here, M. Grammer and J. Godwin (unpublished data) found lower preoptic area AVT mRNA abundances in TP males that did not hold territories relative to TP males that did, but no difference was found between nonterritorial TP males and female-mimic initial-phase males. Together, these findings indicate that the display of dominant male behavior predicts AVT mRNA abundances, whereas gonadal androgen production is not necessary for either increased or sustained production of AVT mRNA. This issue is considered in detail below.

Social, gonadal, and regional differences interact to affect AVT-IR soma size

Instead of a clear effect of dominance, as with AVT mRNA abundances, we found that social status, gonadal status, and POA region interact to influence AVT-IR soma size. AVT-IR soma sizes in the mPOA and pPOA did not vary with treatment, either during sex change or after castration in TP males. However, in the gPOA, intact sex-changing (DS) individuals had significantly larger AVT-IR soma sizes for their body size compared with the DO, SS, and SO groups, indicating a rapid increase in AVT-IR soma size accompanying the assumption of dominance only when testes had developed. Because the absolute AVT-IR soma size (i.e., not corrected for body size) of DS individuals was similar to the much larger TP males of the castration experiment, gonadal input may serve to rapidly increase the size of AVT-IR neurons to that characteristic of fully developed TP males. AVT-IR soma size in the gPOA of TP males is also affected by castration. However, in this case, intact TP males have smaller AVT-IR soma sizes than castrated males.

Bluehead wrasses are not the only teleost fish to display complex interactions between social and gonadal factors regulating neuropeptide neuron size. In another well characterized teleost model, the African cichlid Haplochromis burtoni, the assumption of territorial status in males leads to larger testes, higher 11KT levels, and larger gonadotropin-releasing hormone (GnRH)-IR soma sizes in the POA (Francis et al., 1993; for review, see Fernald, 2002). However, castration of Haplochromis males leads to additional increases in GnRH-IR soma size, whereas androgen replacement reverses these effects (Francis et al., 1993).

Changes in AVT-IR soma size in the gPOA are unlikely to be solely attributable to direct effects of gonadal androgen production. Similar to findings in goldfish (Parhar et al., 2001), exogenous 11KT did not alter the AVT-IR soma size in any POA region in the socially subordinate, ovarietomized females in this study or in intact, laboratory-housed bluehead wrasse females (McIntyre, 1998; cf. Grober and Bass, 2002). Testosterone (T) also appears unlikely to be the cause of these changes, because females have higher circulating T levels than males in many fish, including a congener of the bluehead wrasse, Thalassoma duperrey (Nakamura et al., 1989; for review, see Borg, 1994). Although T and the aromatization of T into estrogen mediate many androgen-dependent sex differences in mammals (De Vries and Simerly, 2002), what little is known about the link between aromatization of T and social status in fish suggests that aromatase may block the masculinization of behavior by preventing the conversion of T to 11KT (Forlano et al., 2001).

Other gonad-influenced systems may link gonadal function to POA cell size. For example, GnRH expression is associated with both sex change and social status [T. bifasciatum (Grober et al., 1991) and H. burtoni (Francis et al., 1993)] and tightly correlated with gonadal function (for review, see Foran and Bass, 1999). Direct links between GnRH and AVT systems have yet to be examined, but interactions between the systems are likely to influence male-typical reproductive behavior (Foran and Bass, 1999). Serotonin and dopamine are also associated with social status through effects on aggression and dominance (Fernald, 2002). Serotonin influences AVP release in hamsters (Ferris et al., 1997), and both neurotransmitters are coregionalized with AVT in the POA of bluehead wrasses (J. Elkins, J. Reed, and Godwin, unpublished data).

The mechanism by which AVT-IR soma sizes in the gPOA are affected is intriguing, because it suggests that different aspects of neuronal phenotype, such as size and neuropeptide production, may be influenced independently. However, the significance of these phenomena in T. bifasciatum is unclear, because differences in AVT-IR soma sizes were not consistently linked to either the increased AVT mRNA abundances (either overall or within the gPOA) or differences in expression of male-typical behavior. Instead, the relative abundances of AVT mRNA did closely correlate with and predict the expression of male-typical behavior. Studies in other fish also suggest a better agreement between AVT mRNA abundance and behavioral expression than with soma size. For example, in Salaria pavo, a fish with reversed sex roles in which females court males, AVT mRNA is expressed in greater abundance per cell in females, despite the fact that males who have higher 11KT levels have larger AVT-IR soma sizes (Grober et al., 2002).

Although the patterns described here are in contrast to those in examples in which the AVP/AVT system is strongly androgen dependent, the regulation of neuropeptide systems should be considered in light of the environment that an animal occupies (Crews and Moore, 1986). Social hierarchies and mating opportunities change quickly and unpredictably for bluehead wrasses. Individuals assuming dominant social roles may have to rapidly increase their display of aggression and territorial defense to suppress these behaviors in other individuals. In bluehead wrasses, the ability to decouple neuropeptide pathways mediating male behavior from testicular function may allow sex-changing females to more rapidly assume dominant behavior and, therefore, suppress sex change and dominant behavior in other individuals.

Together, our results suggest that: (1) unlike in most models, AVT mRNA abundances typical of the dominant male phenotype in bluehead wrasses can both develop and be maintained independent of gonadal inputs, (2) AVT mRNA is a better predictor than AVT-IR soma size of male behavior, and (3) various aspects of neuronal phenotype can respond differentially to hormonal/social signals. Gonad-independent effects (e.g., social influences) on the AVP/AVT system may be common across species but more difficult to discern in many models because of the strong influences of gonadal androgens.
References


CHAPTER IV
MULTIPLE MECHANISMS OF PHENOTYPE DEVELOPMENT IN THE
BLUEHEAD WRASSE

This chapter is currently accepted as:

Semsar, K. and Godwin, J. Multiple mechanisms of phenotype development in the
bluehead wrasses. *Horm. and Behav.*
Abstract

Despite having detailed information on mechanisms mediating sex-typical behavior in many species, we have little understanding of whether the same mechanisms mediate these behaviors when they are performed in the same species under different social contexts. In the five field experiments of this study of bluehead wrasses (*Thalassoma bifasciatum*), a sex-changing fish, we examined the roles of arginine vasotocin (AVT) and the potent teleost androgen 11-ketotestosterone (11KT) in mediating sexual and aggressive behaviors typical of dominant males. We demonstrated that AVT appears necessary for the assumption of dominant territorial status in males and females, but is sufficient only in the socially dominant terminal phase (TP) male phenotype. Specifically, a putative AVT inhibitor prevented both TP males and females from gaining dominance over recently vacated territories. However, unlike TP males, neither females or initial phase males responded to AVT treatment with increases in display of TP male typical behaviors when under social conditions that inhibit sex change. Finally, treating females with 11KT did not alter responsiveness to AVT, but did induce male coloration and courtship behavior that was not observed in oil-treated females. Together these results indicate that the ability of AVT to induce male-typical behavior differs among sexual phenotypes and that this differential responsiveness appears to be mediated by social context and not directly by exposure to 11KT. Furthermore, since 11KT can induce courtship behavior in females that is not affected by AVT, there may be different hormonal mechanisms mediating courtship behavior under different social contexts.
**Introduction**

Sex-typical behaviors are often highly stereotyped, and for many species we now have detailed information about the mechanisms underlying the display of these sex-typical behaviors under specific social contexts. However, the same stereotypical behavior can often be displayed under different social contexts, and we have little understanding of whether sex-typical behaviors performed under variable social conditions are mediated through similar or distinct neural and hormonal mechanisms.

One important character of variable social environments that may influence the mediation of a behavior is the predictability of that social environment or an individual’s social role.

For example, comparisons of species with chromosome-mediated versus environmentally-mediated sex determination highlight the ability of different species to use alternate hormonal mechanisms to develop and express male-typical behavior. In species where sex determination is predictably directed by sex chromosomes, androgens are well-known for playing a key role in the organization and activation of male-typical sexual behavior (DeVries and Simerly 2002). Importantly, one of these roles is to organize the arginine vasopressin/vasotocin (AVP/AVT) system, a key neuropeptide mediator of vertebrate social behavior. As neuropeptides, AVT/AVP can rapidly alter social behavior, often increasing sexual behavior, and therefore may be used by animals to modulate behavioral responses to changing social conditions. The importance of androgens to the regulation of the AVP/AVT system has been demonstrated repeatedly in experiments where castration and/or androgen inhibition decrease both the levels of AVP/AVT and the behaviors the system mediates and are recovered with androgen replacement (reviewed in Goodson and Bass, 2001, DeVries and Simerly, 2002).
However, when sex determination is mediated through environmental signals, gonadal steroids are less dominant in the organization of male-typical behavior and the neural substrates mediating those behaviors. For example, in the leopard gecko *Eublepharis macularius*, a species with temperature-mediated sex determination, temperature directly affects both brain morphology and function (Crews et al., 1996). The bluehead wrasse (*Thalassoma bifasciatum*) is another species in which the environment can directly influence sex-typical brain function without transduction through gonadal steroids. In this sex-changing teleost fish, an individual’s social environment determines its sex as the dominant male sexual phenotype develops in females who become socially dominant (Warner, 1988; Warner and Swearer, 1991). Importantly, social cues can change unpredictably, behavioral sex change can be very rapid, and ovariectomy does not prevent the development of male behavior in socially dominant females (Godwin et al., 1996). Instead, the neuropeptide arginine vasotocin (AVT) appears to play a critical role in the development of territorial male behavior (Semsar et al., 2001; Semsar and Godwin, 2003). Therefore, the bluehead wrasse is a useful model for studying the effects of social environment on the function of neural systems subserving sex-typical social behavior, especially for how the vasotocin system interacts with social cues to mediate appropriate social behavior.

Bluehead wrasse populations include three morphological phenotypes: females, initial phase (IP) males, and terminal phase (TP) males. Females feed throughout the day while hydrating their eggs in preparation of spawning once every 1-3 days. The female-like IP males spawn on a daily basis with groups of other IP males or sneak on pair spawns between the TP males and females. Among the brightly-colored TP males there
are two social classes. Territorial TP (T-TP) males actively defend one of the traditional spawning sites on a reef where it courts and pair-spawns with females during the daily spawning period (approx. 1100 to 1300 hrs; Warner, 1984, 1988). Meanwhile, non-territorial TP (NT-TP) males do not defend a spawning site during the spawning period, moving over large areas of reef and continuing to feed while rarely courting females or chasing conspecifics. However, NT-TP males rapidly occupy territories when these become available. Importantly, manipulation of the AVT system alone can shift social status in TP males. Exogenous AVT can induce territorial behavior in NT-TPs while AVT inhibitors block T-TP males from displaying courtship behavior and guarding their territories and (Semsar et al., 2001).

AVT has also appeared important in the transition from female to male phenotype during sex change. The onset of male-typical behavior in females occurs first, within minutes to hours after TP male removal (Warner and Swearer, 1991). Within two to three days (the earliest time point sampled), increased male-typical behavior is correlated with increased hypothalamic AVT mRNA (Godwin et al., 2000). Furthermore, these increases in AVT mRNA are predictive of the assumption of male behavior regardless of whether females have been ovariectomized or left intact prior to TP male removal (Semsar and Godwin, 2003).

Although behavioral sex change in bluehead wrasses has been linked to changes in AVT, morphological sex change appears directed by androgens. In intact females, testes begin developing 2-3 days after females assume dominance on a spawning site and 2-3 days after the onset of testes development, 11-ketotestosterone (11KT) levels increase (Semsar and Godwin, 2003). Acting analogously to dihydrotestosterone in tetrapods,
11KT mediates male morphological secondary sexual character development in fishes (reviews by Borg, 1994; Grober and Bass, 2002). Specifically in bluehead wrasses, increases in 11KT not only correlate with the development of color change to that typical of the TP morphology in intact individuals (Godwin et al. 1996), but are known to induce color development in ovariectomized females (Semsar and Godwin, 2003). However, despite the importance of 11KT in the development of morphological characters, 11KT is not directly correlated with the assumption of male behavior or increases in AVT mRNA (Semsar and Godwin, 2003).

For this paper, we further examined the role of AVT in the acquisition of territories and the mediation of male-typical behavior under variable social contexts by asking three specific questions. One, is AVT necessary in TP males and/or sex-changing females for the acquisition of newly vacated territories? Two, is AVT sufficient to override social inhibition and induce TP male-typical courtship and social behaviors in IP males and/or females? Three, can exposure to 11KT alter the behavioral responsiveness to AVT in females?

**Methods**

*Field Sites:* Experiments 1 and 4 were conducted during July of 2001 in St. Croix, US Virgin Islands. Experiment 1 was conducted on reefs 6 and 17 while Experiment 4 was conducted on reef 13 (for description of reefs see Gladfelter and Gladfelter, 1987). Experiments 2, 3, and 5 were conducted in April and May of 2002 on patch reefs in Glover’s Reef Atoll lagoon (for description of reefs see Stoddart, 1962).
Experiment 1: AVT effects on territorial acquisition by TP males.

To test whether blocking AVT action also blocks the ability of TP males to assert dominance over newly opened territories, we caught and tagged all TP males with beaded floy tags (for description see Warner, 1988) from two reefs with greater than seven active territories in St. Croix. Tagged TP males were released back on their home reefs the same day and were observed to determine which males were actively defending territories (T-TP males) and which were not (NT-TP males). Within one week of tagging, we removed all T-TP males by lift-netting early in the morning and held in pools at the lab for the remainder of the day. We returned to the reef 80 minutes before the predicted spawning period and gave intraperitoneal injections of either saline or Manning compound (β-Mercapto-β,β-cyclopenta-methylenetriopionyl1, O-Me-Tyr2,Arg8]-Vasopressin, Sigma, St. Louis) (3.2\(\mu\)g Manning/ gram body weight in a 3.2\(\mu\)g Manning/\(\mu\)l saline solution, resulting in injection volumes from 110-165\(\mu\)l (average: 135\(\mu\)l) to all remaining NT-TP males. Manning compound, a specific AVP V1 receptor antagonist, has been shown to effective at this dose of decreasing territorial and mating behavior in T-TP males at this dose (Semsar et al., 2001).

We then observed all sites and all males for evidence of territorial defense. Three observers rotated among three territories each, recording five minute observation bouts of activity on each assigned site. These observers were blind to the treatment of fish. Three to four other observers were assigned to Manning-treated fish without knowledge of the treatments or experiment. Finally, one observer (not blind to treatment) rotated through observers making sure all fish in the experiment were found.
At the end of the spawning period, we had recorded which fish had shown territorial defense of a site at any point of the spawning period. Finally, we placed the T-TP males back on the reef.

Experiment 2: AVT effects in the acquisition of territories during sex change in females.

To test whether blocking AVT would also block the ability of first day sex-changing females to assert dominance over a newly opened spawning site, we caught all IP individuals larger than 45mm from ten small patch reefs with no more than two spawning sites in Glover’s Reef Atoll Lagoon. The largest four to six females were tagged as referenced above and returned to their home reef. All IP males were relocated to distant patch reefs.

The day before a trial, TP males were observed to determine the onset of the spawning period on that reef, and following the spawning period, all TP males were relocated to a distant patch reef. The following day, we gave an intraperitoneal injection of Manning compound ((3.2\text{mg Manning} / \text{gram body weight in a 3.2\text{mg Manning}/ml saline solution}) or saline to the largest female approximately 40 minutes prior to the predicted onset of the spawning period. An observer then recorded courtship, aggression, and inspections in alternating ten minute time budgets. (For a description of behaviors see Semsar et al. 2001). On a given trial day, two reefs in close proximity were used so that a single observer could alternate between reefs. No swim between reefs took more than four minutes.
**Experiment 3: AVT effects in socially inhibited females.**

To test whether AVT could override social inhibition of male behavior in females, we caught and tagged females from small patch reefs with 2-3 spawning sites in Glover’s Reef Atoll Lagoon as described in Experiment 2. In contrast, any IP males smaller than the females were also returned to their home reef while IP males larger than the largest four females were removed. (We only had to remove one IP male from one reef to accomplish this.) All TP males were left on the reef.

Using a paired design (as in Semsar et al., 2001), we tested each female’s response to both saline and AVT treatments. On experimental day 1, each female on a reef was given a single intraperitoneal injection of either saline or AVT (1.0 g/g body weight in a 1.0 mg/10 ml saline solution) and observed in alternating ten minute time budgets for the remainder of the spawning period, recording all behaviors described above. Two days later, on experimental day 3, the same females were given intraperitoneal injection of the opposite treatment they received on experimental day 1. (If a female received AVT on day 1, she received saline on day 3 and vice versa.) The same suite of behaviors was again recorded in alternating ten minute time budgets.

**Experiment 4: AVT effects in IP males.**

To test whether AVT can induce territorial male behavior in IP males, we caught and tagged IP males from Reef 13 in St. Croix as described above. All other individuals were left on the reef unmanipulated. We tested IP males’ responses to exogenous AVT treatment following the same paired design as in Experiment 3. In addition to the
standard suite of territorial behaviors, we also recorded the occurrence of streaking spawns within the group spawn.

**Experiment 5: Interaction between 11KT and AVT in the development of territorial male behavior.**

To test whether differences in phenotypic responses to AVT is due to effects of androgens, we caught, tagged, and ovariectomized the largest 4-8 females (for description see Godwin et al., 1996) from patch reefs with 2-5 TP territories in Glover’s Reef Atoll lagoon. In addition, each female received an 8mm Silastic implant (approx. 20\[\mu\]l) of either castor oil, or castor oil and 11KT (11KT, 5\[\mu\]g/\[\mu\]l, Steraloids, Newport, RI; Silastic tubing, 1.47mmID, 1.96mmOD, Dow Corning, Midland, MI). This dose was chosen based on effectiveness of implants used in goldfish (Stacey and Kobayahi, 1996) and pilot studies in bluehead wrasses showing that ovariectomized females implanted with this dose of 11KT showed full color development within two weeks, similar to natural sex changers which take approximately three weeks (two weeks after full testes development). The implanted females were released the same day onto their home reef and no further social manipulations were performed.

Ten to thirteen days following the surgery, we again used the paired design outlined in Experiment 3 to test whether oil or 11KT treated females would differentially respond to exogenous AVT treatment. To test whether 11KT alone affected behavior, we compared behavior of oil and 11KT-treated females recorded only on days in which individuals received saline. To test whether 11KT affected the responsiveness of females
to respond to AVT treatment, we compared the difference in behavior between the AVT and saline treatments within individuals between the oil and 11KT treated groups.

On the last day of behavioral testing, all individuals were sacrificed and we collected brains and blood samples for further analysis of AVT phenotype and 11KT levels respectively. These results are described in Semsar and Godwin (2003) and referenced in the discussion.

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

**Results**

In Experiment 1, treatment with Manning compound significantly decreased the likelihood that a NT-TP male would become territorial under the permissive social context created by removing T-TP males. Specifically, all nine saline-treated males displayed territorial defense while only one of seven Manning-treated males displayed territorial defense (Fisher exact test, p<0.05; Fig. 1a). Similarly in Experiment 2, treatment with Manning compound significantly decreased the likelihood that a female would become territorial under the permissive social context created when dominant TP males were removed. Specifically, all five saline-treated females displayed territorial and courtship behavior while none of the five Manning compound-treated females displayed any of these behaviors (Fisher exact test p<0.05; Fig. 1b).
Fig. 1. Presence of individuals on site (open bar) and off site (hatched bar) in trials of territorial acquisition with NT-TP males in Experiment 1 (A) and females in Experiment 2 (B).
In neither females (Expt. 3; Table 1) nor IP males (Expt. 4, Table 2) did exogenous AVT treatment administered under inhibitory social contexts significantly change behavior compared to saline controls.

Table 1. Behavioral results of AVT treatment to IP males in Experiment 3. P-values represent two-tailed values of paired t-tests.

<table>
<thead>
<tr>
<th>Behavior</th>
<th>Saline, ave (range)</th>
<th>AVT, ave (range)</th>
<th>p-value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Inspections (no.)</td>
<td>0.375 (0-2)</td>
<td>1.75 (0-7)</td>
<td>p=0.1</td>
</tr>
<tr>
<td>Courtships (no.)</td>
<td>0 (0-0)</td>
<td>0 (0-0)</td>
<td>p=1.0</td>
</tr>
<tr>
<td>Aggressions (no.)</td>
<td>2.25 (0-6)</td>
<td>2.1 (0-6)</td>
<td>p=0.9</td>
</tr>
</tbody>
</table>

Table 2. Behavioral results of AVT treatment to females in Experiment 4. P-values represent two-tailed values of paired t-tests.

<table>
<thead>
<tr>
<th>Behavior</th>
<th>Saline, ave (range)</th>
<th>AVT, ave (range)</th>
<th>p-value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Inspections (no.)</td>
<td>8.7 (1-18)</td>
<td>12.1 (0-25)</td>
<td>p=0.18</td>
</tr>
<tr>
<td>Courtships (no.)</td>
<td>0.09 (0-1)</td>
<td>0.27 (0-2)</td>
<td>p=0.44</td>
</tr>
<tr>
<td>Aggressions (no.)</td>
<td>1.3 (0-5)</td>
<td>1.8 (0-9)</td>
<td>p=0.59</td>
</tr>
<tr>
<td>Group spawns (no.)</td>
<td>4.7 (1-11)</td>
<td>4.8 (0-12)</td>
<td>p=0.93</td>
</tr>
</tbody>
</table>
In Experiment 5, 11KT treatment increased the likelihood an ovariectomized female would show courtship, but not inspections or aggressive behavior on the days of control treatments (Table 3). In addition, there was no effect of exogenous treatment of AVT within either 11KT- or oil-treated females on courtship (paired t-tests, p>0.1; Fig. 2), aggression (paired t-tests, p>0.1; Fig. 3), or inspections (paired t-tests, p>0.1; Fig. 4).

Table 3. Behavioral results of 11KT treatment to ovariectomized females in Experiment 5. P-values represent Fisher exact tests.

<table>
<thead>
<tr>
<th>Behavior</th>
<th>OIL (no./n)</th>
<th>KT (no./n)</th>
<th>p-value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Inspections</td>
<td>2/6</td>
<td>6/9</td>
<td>p&gt;0.1</td>
</tr>
<tr>
<td>Courtships</td>
<td>0/6</td>
<td>5/9</td>
<td>p&lt;0.05</td>
</tr>
<tr>
<td>Aggression</td>
<td>5/6</td>
<td>7/9</td>
<td>p&gt;0.1</td>
</tr>
</tbody>
</table>
Fig. 2. Individual courtship scores for all oil (A; n=6) and 11KT (B; n=9) treated females. Values represent total duration of a behavior from the sum of four ten minute time budgets on the day of treatment (saline or AVT). There is no significant difference between saline and AVT treatment for either oil- (paired t-test, p>0.1) or 11KT- (paired t-test, p>0.1) treated females.
Fig. 3. Individual aggression scores for all oil (A; n=6) and 11KT (B; n=9) treated females. Values represent total duration of a behavior from the sum of four ten minute time budgets on the day of treatment (saline or AVT). There is no significant difference between saline and AVT treatment for either oil- (paired t-test, p>0.1) or 11KT- (paired t-test, p>0.1) treated females.
Fig. 4. Individual inspection scores for all oil (A; n=6) and 11KT (B; n=9) treated females. Values represent total duration of a behavior from the sum of four ten minute time budgets on the day of treatment (saline or AVT). There is no significant difference between saline and AVT treatment for either oil- (paired t-test, p>0.1) or 11KT- (paired t-test, p>0.1) treated females.
Discussion

Our work to date suggests that AVT is both sufficient to induce territorial behavior and dominant-male typical courtship in terminal phase male bluehead wrasses and necessary for the maintenance of these behaviors. The results of this study suggest AVT is also necessary for the development of these behaviors in initial phase individuals becoming T-TP males and for NT-TP males assuming territorial status. However, exogenous AVT is not sufficient to induce territorial behaviors or courtship in initial phase individuals, either females or IP males, under social inhibitory conditions. In addition, 11KT could not induce in females the behavioral responsiveness to AVT that is characteristic of TP males. This result suggests the difference in responsiveness is not solely attributable to androgen environment. The androgen environment can, however, still influence the display of courtship behavior, indicating that there may be multiple neural pathways capable of mediating male-typical sexual behavior in this species.

*AVT action is necessary for territory acquisition.*

In both NT-TP males and sex-changing females competing for open territories, treatment with the AVP V₁ receptor antagonist Manning compound prevented individuals from displaying dominance over a spawning site. These results are consistent with the hypothesis that AVT action is necessary for the acquisition of newly opened territories and in agreement with the results from Semsar et al. (2001) where T-TP males more frequently abandoned their spawning sites after treatment with this same putative antagonist of AVT action. These results complement our previous findings by indicating AVT is necessary for not only the maintenance but also the development of these behaviors and add to the body of information indicating the importance of AVT in sexual
and courtship behavior in general (reviewed in Goodson and Bass, 2001; DeVries and Simerly, 2002).

The ability of individuals to compete for display sites may be a function of the persistence of the display of courtship and aggressive behaviors during the contest for territories. This persistence may, in turn, be mediated by AVT. For example, in grey treefrogs, *Hyla versicolor*, males captured and placed as intruders in other males’ calling sites were more likely to both produce advertisement calls, an AVT-mediated behavior, and take over the calling site from the resident if first given AVT injections (Semsar et al., 1998). In this study, using a receptor antagonist to block AVT-mediated territorial behaviors reduced the likelihood that both NT-TP males and females would secure an open territory. In both cases, the largest and therefore highest socially ranked individuals given Manning compound remained on the reef but did not assume dominance over open spawning sites.

Interestingly, preventing the largest females on the reef from becoming territorial dominants did not result in the display of dominant behaviors by the second largest females. Instead, the spawning site remained unoccupied when the largest female received Manning compound. This indicates that the display of courtship and aggression towards smaller conspecifics by behavioral dominants is not the only factor inhibiting these behaviors in smaller females. This is supported by studies in other sex-changing species where larger individuals prevented from interacting directly with smaller individuals by mesh barriers can still suppress sex change in the smaller individuals (*T. duperrey*, Ross et al., 1983; Lutnesky, 1994; Morrey et al., 2002).
**Sufficiency of AVT treatment is dependent on sexual phenotype**

While the response to an AVT inhibitor is consistent with the hypothesis that AVT action is necessary for the display male-typical behavior, there is a marked difference between phenotypes in the ability of exogenous AVT treatment alone to induce these behaviors. Specifically, exogenous AVT treatment of females and IP males on the reef in the presence of T-TP males, a social environment that inhibits sex and role change, did not induce the display of any TP male-typical behaviors. Indeed, females continued to spawn as females and IP males continued to spawn in the group spawns. In contrast, our earlier work showed that AVT treatment of NT-TP males was sufficient to induce the display of territorial and courtship behavior (Semsar et al., 2001). AVT-treated NT-TP males would court and defend a novel area on a reef even in the presence of T-TP males who were occupying all of the established spawning sites. Therefore, AVT is sufficient to induce the display of male-typical behaviors only in the dominant TP male phenotype and not in either of the subordinate phenotypes, female and IP male.

Several potential mechanisms could account for this difference in responsiveness to AVT treatment between phenotypes. These include differences in dosages necessary to elicit a response, differences in responses to handling and injection (potential interaction with cortisol responses), and differences in AVT receptor abundances and/or distribution. Although we have no data to directly address the first two points, we do have preliminary data suggesting that TP males have higher AVT receptor abundances than females (unpublished data) and are further exploring this possibility.

Our results suggesting that the effects of exogenous AVT depend on sexual phenotype agree with results in another teleost fishes. For example, in the plainfin
midshipman (*Porichthys notatus*), a species with fixed alternative male phenotypes, AVT alters the production of fictive vocalizations in territorial type I males but not in non-territorial type II males and females (Goodson and Bass, 2000). Again, in the weakly electric fish (*Apteronotus leptorhynchus*), AVT modulates chirping behavior used in both sexual and aggressive contexts in males but not females (Bastian et al. 2001). Bluehead wrasses exhibit extreme phenotypic plasticity, including the ability to switch from female to male behavior within hours. On this basis, we might predict that they would more likely to mediate sex-typical behaviors through similar mechanisms across sexual phenotypes. However, we have no evidence to suggest this is the case. Despite the differences in plasticity, the differential responsiveness of the sexual phenotypes in these species are similar with exogenous AVT only being behaviorally effective in the territorial male phenotype but not the female or female-mimic male. It is also noteworthy that sex differences in the behavioral effectiveness of neuropeptides are observed with phenomena such as partner-preference formation in voles, where AVP is only effective in males and oxytocin is only effective in females (reviewed in Insel and Young, 2000).

*Interaction between 11KT and AVT systems*

Considering the differential responsiveness between sexual phenotypes to exogenous AVT treatment, we sought to test what factors might underlie this difference. One obvious difference between the female and TP male phenotype is the presence of high circulating levels of 11KT, the main teleost androgen known to be responsible for the development of morphological secondary sexual characteristics, including the difference in color patterns in bluehead wrasses (Godwin et al., 1996; Semsar and Godwin, 2003) as
well as many other sex-changing and non sex-changing fishes (Hourigan et al., 1991; Cardwell and Liley, 1991; Brantley et al., 1993; Borg, 1994). Despite their necessity for color patterns, neither gonads nor high circulating 11KT are necessary for the display of AVT mRNA expression and the administration of 11KT to females does not increase AVT mRNA expression compared to oil-treated controls (Semsar and Godwin, 2003; see also Parhar et al., 2001 for similar results in goldfish). However, 11KT could still play a role in the responsiveness to exogenous AVT treatment by affecting the number/distribution of AVT receptors or through interactions with other neural signaling systems.

Neither of these possibilities is supported by our experiments, however. Although females treated with 11KT did reliably develop secondary sexual characteristics such that they looked like NT-TP males on the reef (see Semsar and Godwin, 2003), they did not respond to exogenous AVT treatment with any discernible change in behavior. This sharply contrasts our previous findings in NT-TP males where (as noted above) exogenous AVT increased the display of inspections, courtship, and aggression and defended a novel site on the reef as if it were a traditional spawning site (Semsar et al., 2001). It should be noted that other studies examining the effects of androgen implants on the development male phenotypes have tested for longer time periods (e.g., Stacey and Kobayashi, 1996). However, the AVT responsiveness we see in bluehead wrasses can occur in late stage transitional males and this stage can be reached approximately two weeks after 11KT levels increase. This argues that the 11KT implants should have been in the fish enough time to detect effects. Although it is still possible that difference in release rates between naturally developing TP males and 11KT-implanted females could
account for the lack of apparent organization of AVT responsiveness, the phenotypic differences in the effectiveness of AVT to override social cues does not appear due to be solely driven by production of 11KT.

That 11KT had no effect on the ability of AVT to induce territorial behavior or courtship displays suggests that putative inhibition of the AVT system by some aspect of social status was still operative. Social rank appears to be determined by body size relative to other individuals. Since hormone-treated females were placed back into their original social groups, their social rank therefore may have remained unchanged.

Experiments in a congener Thalassoma duperrey that has a very similar social structure to that of T. bifasciatum have shown that social rank as determined by relative body size is a key environmental mediator of sex change. Larger individuals suppress sex change, smaller individuals stimulate sex change, and the relative body size of a fish compared to others in its social group is the key predictor of the occurrence of sex change (Ross et al., 1983, 1990).

What neural mechanisms might link social rank to AVT responsiveness? Two possibilities have been put forward. A set of recent experiments have demonstrated that changes in monoamine activity, specifically serotonin, dopamine, and norepinephrine are correlated with these changes in social rank and sex change (Larson et al., 2003a). Indeed, manipulations of these systems can override the effects of social rank to induce and suppress sex change under inhibitory or permissive social conditions respectively (Larson et al., 2003b).

Indeed, interactions of the AVP system with the serotonergic system can play key roles in mediating dominance relationships (e.g. Ferris et al., 1997; Delville et al., 1998).
and this may explain why 11KT-treated females in this study were unresponsive to AVT treatment. If inhibitory effects of monoamines such as serotonin are maintained by subordinate ranking in an individual’s social group, social inhibition of the 11KT-treated females placed back into their original social group would have been maintained. This hypothesis is consistent with the results that neither responsiveness to AVT treatment (Expt. 5) or AVT mRNA expression (Semsar and Godwin, 2003) changed with 11KT treatment. Therefore, one potential mechanism critical for the display of TP male-typical courtship behavior may be the release of inhibition by monoamines on the AVT system through changes in the position of an individual in its social rank. Once inhibition has been removed, the AVT system may then directly mediate TP male-typical behavior.

A second model relating social rank to sex change proposes that aggression from territorial TP males places females and IP males under chronic social stress and this, in turn, generates elevations in circulating cortisol levels that inhibit sex change (Perry and Grober, 2003). This model has not been directly tested in a female-to-male sex-changing fish. However, in a male-to-female sex-changing fish, *Amphiprion melanopus*, cortisol levels do not differ between males and females, but rather increase over the course of sex change (Godwin and Thomas, 1993).

*Multiple neural mechanisms of behavioral phenotype development*

Interestingly, although 11KT did not alter the effectiveness of AVT treatment, 11KT treatment alone was enough to induce the display of opportunistic courtship in females. Over half the females given 11KT were observed displaying courtship behavior on days they received saline injections. All these courtship attempts were directed
towards females located in the feeding school on the upcurrent side of the reef or over a T-TP’s spawning site while it was away from that site. This behavioral profile is typical of courtship seen in untreated NT-TP males but not of NT-TP males treated with AVT (Semsar et al., 2001). Once again, in the context of species with fixed phenotypes and androgenic organization of male behavior, the induction of male behavior following androgen treatment would not be surprising (DeVries and Simerly, 2002). However, in bluehead wrasses where gonads and gonadal androgens are not necessary for the display of male behavior (Godwin et al., 1996; Semsar and Godwin, 2003), this was a surprising result. The induction of TP male-typical courtship behavior with 11KT treatment is especially interesting in light of the fact that 11KT treatment had no effect on AVT mRNA levels in any area of the POA (Semsar and Godwin, 2003). Therefore, for the first time in our experiments with bluehead wrasses, we saw variable behavioral phenotypes that were not correlated with differences in AVT mRNA expression. Importantly, the context in which these behaviors were expressed was very different than previous experiments (i.e., under social inhibition rather than under socially permissive conditions for sex change). Therefore, we propose that there may be multiple neural mechanisms that underlie courtship behavior in bluehead wrasses and that the different mechanisms subserve these behaviors under different social contexts.

Although the phenotypic plasticity seen in sex-changing fish is dramatic, the existence of multiple neural mechanisms for critical behaviors may be common. For example, numerous studies of knockout mice have demonstrated that when one neural mechanism is no longer functional, other systems may compensate for the loss (Bilbo and Nelson, 2001). Compensation during development has also been shown in more classical
hormonal manipulations affecting the vasopressin system. In male rats, the AVP system is normally both organized and activated by androgens and many male-typical behaviors, including social recognition, are AVP-dependent (DeVries and Simerly, 2002). Interestingly, Axelson and colleagues (1999) showed that in utero treatment with flutamide, an inhibitor of androgen synthesis, did not eliminate social recognition in male rats, but did eliminate the dependence of this process on AVP.

Although these compensation mechanisms are often interpreted as redundancies in the developmental pathways for critical behaviors, the ability to maintain the expression of behavior and compensate for deficits during development is unlikely to be the only way in which having multiple neural mechanisms for the display of a given behavior is important. Indeed, these mechanisms of developmental compensation may also provide us with windows into sources of plasticity available to adult individuals to modulate behavior in different social conditions. Further exploration of mechanisms operating in different social contexts should improve our general understanding of behavioral adaptation.

**Conclusion**

AVT appears necessary for territory acquisition in both TP male and female bluehead wrasses. The sufficiency of AVT to induce male-typical behavior under social inhibition is, however, dependent on sexual phenotype with TP males sensitive to AVT treatment and females and female-mimic (IP) males insensitive to treatment at similar doses. This difference in responsiveness to AVT treatment among phenotypes appears to be mediated by social context rather than the direct action of 11KT, the potent teleost
androgen. Finally, 11KT can induce courtship behavior in females without apparent changes in AVT action indicating that there may be different hormonal mechanisms mediating the onset of courtship behavior under different social contexts.
Appropriate timing of reproductive behavior is critical to reproductive success. For seasonal breeders, this often results in animals cycling through multiple life history stages as adults, with at least two stages, breeding and non-breeding seasons (e.g. Wingfield et al. 1992). Other species, including sex-changing fish, transition between alternate phenotypes is key to maximizing reproductive success (Ghiselin 1969, Warner 1984). In any of these cases, transitions between phenotypes are generally orchestrated by hormones in response to various types of environmental cues. Understanding how nature of how hormones can orchestrate such phenotypic change requires an understanding of what environmental cues provide predictive information about the appropriate timing of reproduction and how these cues are transduced by the animal.

Based on models of information theory, Wingfield and colleagues (1992, 1993) have developed a model to examine how animals use environmental cues to appropriately time the onset of breeding. Although developed in the context of avian life history stages, the model may be applicable to more generalized changes in phenotype which are directed towards optimizing reproductive success. The goal of this chapter is compare the signal-hormone-behavior relationships in the model described by Wingfield et al. (1992, 1993)

Central to Wingfield’s model is the integration of various types of environmental signals, weighting the reliance an animal has on any one signal by how accurately that
environmental signal predicts optimal breeding success. In environments where optimal breeding opportunities are predictable, animals are expected to rely on few, long-term predictive cues (initial predictive cues), such as photoperiod, to time breeding. In contrast, animals living in environments where optimal breeding opportunities are less predictable should integrate both initial predictive cues and more fluctuating environmental cues (termed supplemental cues), such as temperature, food resources, or social cues, that provide additional information about when breeding is most likely to be successful.

The degree to which these initial predictive cues are effective alone or are best integrated with supplementary cues will be determined by whether these cues are enough to predict maximum breeding success (Wingfield et al. 1992, 1993). Furthermore, these environmental cues are often translated through the endocrine system and different types of cues may be translated through different types of endocrine pathways. Long term cues such as photoperiod are known to induce changes in gonadal steroid production which can then act on neural substrates of behavior either directly or indirectly. When photoperiod alone can accurately predict optimal breeding success, gonadal hormones will likely activate breeding behaviors directly. For example, in arctic birds where optimal breeding is gonadal stimulation by photoperiod tightly controls breeding behavior. However, if photoperiod alone cannot predict optimal breeding success, gonadal hormones may act more permissively to prepare an individual to respond to supplementary cues. These short term cues may best be mediated directly through faster mechanisms, e.g. neurotransmitter changes, which serve to inhibit or activate reproductive behaviors rapidly in response to changing environmental conditions.

Therefore, understanding life history characters and the types of environmental cues a species using to make decisions about timing breeding may also determine of the types of endocrine functions that most directly influence reproductive behavior (Wingfield and Silverin 2002).

Although bluehead wrasses breed continuously throughout the year, it does change sexual phenotype to maximize reproductive success. While females spawn at best once a day, T-TPs can spawn up to 70 times a day (on average ~30 per day) year round. This great discrepancy in reproductive success between TP males and females has driven the evolution of female-to-male sex change in this species (Ghiselin 1969, Warner 1975, Warner et al. 1975, Warner 1984). Within this natural history strategy, maximal lifetime reproductive success for any individual is contingent on growing large, becoming male, and gaining dominance on a traditional spawning site (Warner 1978, Warner 1988).

Gaining dominance on one of the limited spawning sites requires being one of the largest individuals on the reef and almost always, all TP males on the reef are larger than the females. Therefore, for a female to eventually gain access to a spawning site, she likely needs to first switch to the TP male phenotype where ‘she’ can grow to a competitive size (Warner 1984). Based largely on research in a congener T. duperrey, this most likely occurs due to a change in the ratio of smaller and larger individuals on the reef (Ross et al. 1983). Relative size in the social group is predictable and probably a reliable cue for assessing the likelihood of gaining dominance over a spawning site. For example, if there are seven spawning sites on a reef and as a hypothetical female you are the 9th largest fish (male and female), by the time you have completed sex and color
change, becoming an NT-TP male, a spawning site is likely to become available shortly (since the average life span of a TP male is approximately three months, Warner 1988). Therefore, social rank may be the initial predictive cue for bluehead wrasses, analogous to photoperiod for seasonally breeding species. It then follows that social rank within the hierarchy may elicit androgen mediated mechanisms to organize and activate the opportunistic courtship and TP coloration associated with the NT-TP phenotype. This switch to the NT-TP phenotype mostly likely prepares a TP male to assume dominance of a territory immediately when one becomes available. In this light, territory availability/occupation is likely to act as a supplementary cue.

This natural progression through life history stages is critical to understanding the mechanisms by which AVT mediates courtship and territorial behaviors in the bluehead wrasse, especially since the social manipulations we often use do not mimic this process precisely. In order to study the mechanisms of sex change with some control of the social environment in nature, initial manipulations of the social environment induced sex change by removing all males at once (Warner and Swearer 1991; Godwin et al. 1996, Godwin et al. 2000, Chapter III). This experimental paradigm effectively changes both social rank and territorial availability at the same time. Therefore, hormonal mechanisms that underlie the responses to these different environmental cues are occurring simultaneously. Consequently, even if these mechanisms mediating sex and behavior change are often or usually distinct from one another under natural sex change conditions, they would be difficult to distinguish using this experimental design. As a result, data obtained from experiments that allow direct development of females into T-TP males must be analyzed cautiously with respect to the detailed relationships between
specific environmental cues and hormonal mechanisms regulating different aspects of phenotype change. For example, in data from sex-change experiments in which females direct develop into T-TP males (Chapter III and IV), we can confidently state that AVT mediates male behavior in the context of gaining access to a spawning site. However, we cannot determine whether AVT is mediating male behavior in response to a change in social rank, a change in territorial availability, or both, since both changed simultaneously.

Keeping natural phenotype progression in mind is especially helpful when interpreting our data on 11KT treated females (Chapter IV). These females were placed back on the reef in the same social ranking. This separated the effects that social rank has directly on the brain and behavior from those mediated by the gonad. By giving 11KT to females, we maintained the direct effects of social inhibition intact while simulating the effect of 11KT production that occurs as consequence of sex change. This experiment resulted in the expression of opportunistic courtship behavior without territorial behavior and without any discernible changes in either AVT neural phenotype or behavioral responsiveness to exogenous AVT treatment (Chapters III and IV). From this we can conclude that 11KT and AVT alone or in combination are not enough to override inhibition of social rank on the AVT system. Therefore, another hormonal signal such as cortisol or serotonin (see Chapter IV for detailed discussion) must be mediating environmental cues associated with social rank.

Additionally this experiment demonstrated that AVT is not the only mediator of courtship behavior. 11KT, or perhaps other hormones it interacts with, can mediate courtship behavior outside the context of territorial dominance (opportunistic courtship).
Since AVT can mediate courtship behavior in contexts of territorial dominance, there appear to be multiple mechanisms that may mediate courtship behavior under varying social contexts in this species. At first this appears to contrast with findings in song sparrows, *Melospiza melodia morphna*, where the regulation of territorial aggression under variable social contexts appears to be under similar control (Wingfield and Soma 2002). In song sparrows, territorial aggression both inside and outside the breeding season appears to be controlled through the production of estrogen despite the fact that the endogenous delivery method of testosterone to the brain differs between contexts (Soma et al. 2000; Soma and Wingfield 2002). During the breeding season when circulating testosterone is elevated, gonadal testosterone mediates aggression (Wingfield 1994). However, outside the breeding season, when gonads are regressed and circulating testosterone is low, adrenal DHEA, an inactive precursor to testosterone, mediates aggression (Soma and Wingfield 2002).

When comparing these systems there are several differences that may account for the different patterns of hormone-behavior relationships. For example, in song sparrows, it is unknown to what degree neuropeptides like AVT aid in mediating territorial aggression or whether there are seasonal differences in potential effects. Conversely, in bluehead wrasses, we have only studied 11KT to date, an androgen that cannot be aromatized, and we do not know what effects testosterone or its metabolites may be having on these behaviors. However, an intriguing and perhaps important difference between the ecology of these hormone-behavior mechanisms is the type of environmental cues that are being used. In the song sparrow, the behavioral responses in both social contexts are to supplemental, social cues, that of simulated territorial intrusions.
(Wingfield and Soma 2002). In other words, the behavior being tested is a reaction to a specific event, regardless of the breeding context. Meanwhile, in the bluehead wrasse, the multiple mechanisms we refer to result from varying types of cues, i.e. changes in social rank, an initial predictive cue, and changes in territorial availability, a supplementary cue. As more research is done on the integration of environmental signals and their underlying mechanisms in these and other species, it will be interesting to see if any additional trends are seen that suggest similar hormone-behavior mechanisms for responses to the same types of signals under varying social contexts and multiple mechanisms for responses to different signals under varying social contexts. If we can find such relationships in hormone-behavior relationships across species, we will greatly increase our power to predict hormone-behavior relationships.
LITERATURE CITED


