PROXIMATE AND DEVELOPMENTAL MECHANISMS OF SOCIAL BEHAVIOR IN THE ZEBRA FINCH

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PROXIMATE AND DEVELOPMENTAL MECHANISMS OF SOCIAL BEHAVIOR IN THE ZEBRA FINCH

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An integrative understanding of the evolution of complex social behavior requires a framework that links insights about the ecological and phylogenetic context of behavior, with the molecular, neural, and developmental mechanisms that produce it. In order to provide insight into the mechanisms underlying complex adaptive social behaviors, I examined the proximate and developmental factors that contribute to species-typical social behaviors in a well-studied song bird, the zebra finch (*Taeniopygia guttata*). Zebra finches demonstrate selective affiliation between juvenile offspring and parents which, like affiliation between pair partners, is characterized by proximity, vocal communication and contact behaviors. In addition, they exhibit vocal learning, in which juvenile males learn courtship song through socially-guided feedback from adult tutors. I demonstrate that proximate factors—including age, breeding experience, and the social group—influence pairing, reproductive success, and the flexible use of alternative reproductive strategies in the zebra finch. Additionally, I present the results of an experiment testing the hypothesis that the nonapeptide arginine vasotocin (AVT, avian homologue of vasopressin) and nonapeptide receptors play organizational roles in the development of species-typical affiliative behavior, courtship, and vocal learning. Zebra finch hatchlings of both sexes received intracranial injections (posthatch days 2-8) of AVT, Manning Compound (MC, a V1a receptor antagonist) or a saline control. I assessed affiliative behaviors using a series of

behavioral assays throughout development. I demonstrate that manipulations of the AVT system early in life alter affiliative interest in parents and opposite sex conspecifics during juvenile development as well as vocal learning in males. I also provide the first evidence that AVT and nonapeptide receptors play organizational roles in both the development of pair bonding in adulthood and the neural substrate underlying these behaviors in a bird. Thus, my research provides support for the idea that the nonapeptides, which modulate the activity of neural circuits across different social contexts, may provide an important mechanism underlying both the evolution and the development of diverse social phenotypes across vertebrate taxa.

BIOGRAPHICAL SKETCH

Nicole Marie Baran is a native of Colorado. After graduating from the International Baccalaureate Program at Lakewood High School in 2005, she attended the University of Chicago in Chicago, IL, where in 2009 she received a Bachelor of Arts, double majoring in Comparative Human Development (specializing in Comparative Behavioral Biology) and Economics. While at the University of Chicago, Nicole worked for two and half years on an interdisciplinary and collaborative project investigating the psychological, hormonal, and behavioral predictors of success in M.B.A. students led by the behavioral biologist Dario Maestripieri, Professor in Comparative Human Development, Evolutionary Biology, and Neurobiology at the University of Chicago, and two economists, Luigi Zingales, Professor of Finance at the University of Chicago Booth School of Business and Paola Sapienza Professor of Finance at the Northwestern University Kellogg School of Management.

In September 2009, she entered the graduate program in Psychology at Cornell University where she began research into the mechanisms of success a new class of vertebrates: birds! Working in the lab of Elizabeth Adkins-Regan, she has studied a wide range of complex social behaviors in birds, including the affiliative behavior and the flexible use of alternative reproductive strategies in birds, including extra-pair copulation, conspecific brood parasitism, sex ratio adjustment, and sex allocation. To support her empirical research, she has also developed two mathematical models in collaboration with H. Kern Reeve: a game theory model of conspecific brood parasitism and a dynamic state variable model of hormonal pleiotropy.

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CHAPTER ONE

Introduction

An integrative understanding of the evolution of complex social behavior requires a framework that links insights about the ecological and phylogenetic context of behavior, with the molecular, neural, and developmental mechanisms that produce it. In order to provide insight into the mechanisms underlying complex, adaptive social behaviors, I examined the proximate and developmental factors that contribute to species-typical social behaviors in a well-studied song bird, the zebra finch (*Taeniopygia guttata*). In Chapter two I present the results of an experiment demonstrating that proximate factors—including age, breeding experience, and the social group—influence pairing, breeding, and the flexible use of alternative reproductive strategies in the zebra finch. In Chapters three, four, and five, I present the results of an investigation testing the hypothesis that the nonapeptide arginine vasotocin (AVT, avian homologue of vasopressin) and the nonapeptide receptors play organizational roles in the development of social behaviors in the zebra finch. In Chapter three, I present results demonstrating that intracranial (IC) injections of AVT or a nonapeptide receptor antagonist early in life influence attachment and affiliation with parents in juvenile zebra finches and their interest in opposite sex conspecifics as they reach reproductive maturity. In Chapter four, I show that AVT and nonapeptide receptors exert organizational effects on the neural pathways involved in species-typical pair bonding behavior in adult male zebra finches. In Chapter five, I present the results of two experiments demonstrating that manipulations to nonapeptide circuitry early in life also influence vocal learning in the zebra finch. Finally, in Chapter six, I argue that the development of the nonapeptide system, which can modulate the activity of specific neural circuits in different social contexts, may provide an

important mechanism underlying both the evolution of diverse social phenotypes across vertebrate taxa.

Theoretical and Methodological Approach

This research is grounded in the assumption that social behavior can have multiple causes, operating simultaneously at multiple levels of analysis (Mayr, 1961; Tinbergen, 1963). Social behaviors serve important functions in the life of an organisms. Social behaviors, like all phenotypic traits, have been subject to selection over in the course of evolution, but we can also partially account for the existence of many behavioral traits by understanding the phylogenetic relationships among organisms. An organism's evolutionary history can thus constrain its evolution, and awareness of this evolutionary context frequently provides insight into the adaptive significance of behavioral traits. However, social behaviors also have more proximate or immediate causes, which can include the neural or neuroendocrine state of the organism, the organism's developmental experience, and proximate social factors.

This research aims to disentangle the multiple proximate causes underlying complex, adaptive social behaviors (Laland et al., 2011; Lehrman, 1970; Odling-Smee et al., 2003). Using an integrative and interdisciplinary approach, I conducted my research in a song bird species that is well-suited to contribute to our understanding of both the diversity of social phenotypes found in the natural world and the diversity of mechanisms that can be used to produce them. I seek to understand the multiple mechanisms by which adaptive social behavior can develop and evolve.

This research employs techniques and philosophical frameworks from multiple disciplines, including behavioral neuroendocrinology, developmental psychobiology, behavioral ecology, and evolutionary-developmental biology. I use an experimental approach to test the effect of multiple

proximate factors (including breeding experience, age, and group composition), as well as developmental factors on ecologically-relevant social behaviors and reproductive outcomes in zebra finches.

Study System

Although not explicitly comparative, this work is motivated and informed by the ecological and evolutionary context of social behaviors in the chosen study organism. Zebra finches are small estrildid finches native to Australia. They inhabit shrubby habitats in arid interior of the continent, breeding opportunistically in response to unpredictable rainfall (Zann, 1996; Zann et al., 1995). Zebra finches exhibit many adaptations which allow them to survive in a harsh and highly unpredictable environment. In the wild, zebra finches experience very high rates of mortality: in one population the mean annual survivorship for the first 12 months of life was only 4% and zebra finches have a high rate of clutch failure, both in natural and captive populations (Fenske & Burley, 1995; Millam et al., 2001; Zann, 1996).

Like more than 90% of bird species, they exhibit socially monogamous pair bonds in adulthood (Bennett & Owens, 2002; Immelmann, 1972; Zann, 1996). Adult zebra finches are highly motivated to form pair bonds and are always paired, even when they are not actively breeding. These pair bonds are permanent rather than short term, which allows them to be prepared to initiate reproduction when environmental conditions are suitable (Adkins-Regan & Tomaszycki, 2007). The pair relationship is characterized by constant physical proximity, frequent vocal communication and contact behaviors, such as clumping (perching in contact), allopreening (mutual grooming), and spending long periods of time together inside of a nest.

Together, both members of the pair equally participate in parental care, which includes nest building, incubation of eggs, and the brooding and feeding of chicks (Zann, 1996). Zebra finch pairs are characterized by mutual investment in parental effort. Close coordination and cooperation between the pair partners has been found to lead to higher reproductive success (Mariette & Griffith, 2012; Schuett et al., 2011). Unlike many species subject to more extensive sexual selection, zebra finch pairs form as a result of mutual mate choice. For example, zebra finch males exhibit choice and tend to court more fecund females (Jones et al., 2001; Monaghan et al., 1996) and both male and female mate preferences are repeatable (Holveck et al., 2011).

The average brood size is 4 chicks (Zann, 1996). Chicks rely on parental incubation for thermoregulation for at least the first 7 days of life. The young fledge around day 18 post-hatching, but remain dependent on parental feeding until approximately 35 days of age (though they may remain in contact with parents for longer than 48 days) (Boogert et al., 2014; Zann, 1996). Within a day or two of fledging, the young are led away from the natal nest by the father to a roosting nest (Zann, 1996). As a result, the juveniles begin to spend more time with other young birds and away from the parents.

Courtship behaviors develop gradually. Male zebra finches, similar to many song bird species, learn courtship song via socially-guided feedback from adult tutors (Eales, 1985; Jones et al., 1996; Slater et al., 1988). Indeed, song learning in zebra finches has become a model system for understanding general principles underlying complex vocal learning across species, including language learning in humans. Over the course of development, male zebra finches learn to produce a song that closely resembles the song produced by their social father which is then used both in mate attraction and the maintenance of the pair relationship (Zann, 1995). Zebra finches learn exclusively during an early sensitive period, which lasts from approximately day 25 to 90 post-

hatch (Slater et al., 1988). Early in this sensitive period, males produce "subsong," which progresses into more mature and structured plastic song, before ultimately "crystallizing" in the final adult song at approximately 120 days post hatch. In zebra finches, this crystallized song is highly stereotyped, and does not change during adulthood. By about seven weeks of age, courtship is directed exclusively towards members of the opposite sex, but it is generally rare in its full form until about 60 days post-hatch in females and day 90 in males.

In addition, zebra finches have been found to use alternative reproductive strategies, such as conspecific brood parasitism and extra-pair mating, in both field and captive populations, with drastic variation in the rates between different environments (Birkhead et al., 1990; Burley et al., 1996; Forstmeier et al., 2011; Griffith et al., 2010; Tschirren et al., 2012; Schielzeth & Bolund, 2010). This suggests that zebra finches have the capacity to exhibit great deal of flexibility in reproductive behaviors, making them amenable to research investigating specific environmental and physiological factors that may underlie such behavioral plasticity.

There are several reasons zebra finches are an ideal system in which to investigate the proximate mechanisms of social behaviors. First, zebra finches are very social and are socially-housed in our lab, permitting the study of their natural social behaviors within a controlled environment. Second, their ecology has been well-studied in the wild, which allows us to situate insights about the mechanisms of their behaviors within the appropriate ecological and evolutionary context. Thus, the zebra finch has become a model organism, commonly studied for its social and reproductive behavior in the lab (Griffith & Buchanan, 2010). Third, zebra finches also breed well in captivity and have a remarkably fast life cycle for a vertebrate, making developmental investigations tractable in this species. Finally, as a model organism in the field of

neuroscience with a fully-sequenced and well-annotated genome, zebra finches are ideal for connecting behaviors to their neural mechanisms.

Scientific Significance

A major scientific challenge is to identify the mechanisms underlying the evolution of species differences in social behaviors. However, progress in this domain depends upon a foundational understanding of how multiple, interconnected mechanisms influence the nervous system across a wide range of species and across time. Taking a comparative approach, this research investigates the proximate mechanisms social behavior in a song bird, with an emphasis on the role of development.

The mechanisms that allow zebra finches to exhibit such complex and flexible social behavior in the appropriate context is still poorly understood. In fact, we still understand very little about the neural and neuroendocrine mechanisms of pairing in birds, despite the fact that more than 90% of bird species exhibit socially-monogamous pair bonds (Bennett & Owens, 2002). The only vertebrate species for which significant progress has been made in finding mechanisms for pairing is the prairie vole (McGraw & Young, 2010). However, the specific details of the prairie vole findings do not generalize to all socially monogamous species [marmosets (*Callithrix penicillata*), Smith et al., 2010; convict cichlids (*Amatitlania nigrofasciata*), Oldfield & Hofmann, 2011; zebra finches, Goodson et al., 2004]. Thus, our understanding of the general principles of how brains have evolved to produce diverse social phenotypes, is limited by our lack of understanding of the diversity of mechanisms that are possible. Only by surveying a wider array of species that appropriately reflect the remarkable diversity of life, can we confidently infer general principles governing variation in sociality (Hofmann et al., 2014).

Furthermore, there is a lack of research that directly links the developmental changes in social behaviors, such as attachment and affiliation, to their neural and neuroendocrine mechanisms in any species. However, evidence suggests that the neural substrate underlying species-typical affiliative behaviors are established early in development. Thus, if we hope to understand the evolution of affiliative behavior generally, we must understand how it develops. Furthermore, there is reason to believe that there are several 'critical periods' during development, during which the developing organism is particularly sensitive to environmental perturbations which may lead sometimes to adaptive, but occasionally pathological, behavioral phenotypes later in life. The role of early experiences in shaping later social behaviors has been well studied in humans, and more recently modeled in rats (Ainsworth, 1989; Champagne et al., 2003). However, the development of the neurobiology underlying affiliative behavior is not well understood in any species that demonstrates attachment behaviors in adulthood.

Thus, this dissertation advances understanding on two fronts, contributing both to our understanding of the diversity of mechanisms that mediate social behavior and how social behaviors develop. This work has led to many novel contributions to our understanding of the mechanisms of social behavior. First, these results provide evidence that age and breeding experience play important roles in the flexible use of facultative and adaptive reproductive strategies in female zebra finches. However, the developmental work, in particular, has the potential to open up an entirely new line of inquiry into the role that nonapeptides play in both the evolution and development of social behaviors. These results are the first demonstration that AVT and nonapeptide receptors play organizational roles in social behaviors in any bird species. These findings also represent the first evidence that manipulations of the AVT system early in life can alter a whole suite of social behaviors in zebra finches, including attachment to the parents, interest

in opposite sex conspecifics, and pair bonding. These results are the first to show that these manipulations alter the neural substrate of social behaviors in adulthood, changing later sensitivity to nonapeptides and the activity of neurons in structures that are involved in the contextually-appropriate expression of behaviors. Finally, these results provide the first evidence that the nonapeptides play a causal role in vocal learning, a complex, species-typical behavior.

In summary, I use an integrative and interdisciplinary approach to understand the multiple factors contributing to the expression of social behavior in the zebra finch, spanning multiple levels of analysis. By connecting the neural, developmental and proximate social mechanisms to the expression of adaptive social behavior, I have contributed important novel insights which have significantly increased our understanding of how the social brain evolves.

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CHAPTER TWO

Breeding experience, alternative reproductive strategies and reproductive success in a captive colony of zebra finches (*Taeniopygia guttata*)

Abstract: Birds exhibit a remarkable diversity of different reproductive strategies both between and within species. Species such as the zebra finch (*Taeniopygia guttata*) may evolve the flexible use of alternative reproductive strategies, as well as benefit from prior breeding experience, which allow them to adaptively respond to unpredictable environments. In birds, the flexible use of alternative reproductive strategies, such as extra-pair mating, has been reported to be associated with fast reproduction, high mortality and environmental variability. However, little is known about the role of previous breeding experience in the adaptive use of alternative reproductive strategies. Here we performed an in-depth study of reproductive outcomes in a population of domesticated zebra finches, testing the impact of prior breeding experience on the use of alternative reproductive strategies and reproductive success. We provide evidence that older females with prior breeding experience are quicker to initiate a clutch with a new partner and have increased success in chick rearing, even in a captive colony of zebra finches with minimal foraging demands. We also find evidence that the breeding experience of other females in the same social group influences reproductive investment by female zebra finches. Furthermore, we demonstrate that the use of alternative reproductive strategies in female zebra finches is associated with previous failed breeding attempts with the same pair partner. The results provide evidence that age and breeding experience play important roles in the flexible use of both facultative and adaptive reproductive strategies in female zebra finches.

Introduction:

The remarkable diversity of avian reproductive strategies is thought to be determined by variability in ecological resources, opportunities for exploiting social or sexual partners and by phylogeny (Bennett & Owens, 2002). A majority of birds form socially monogamous pair bonds and exhibit biparental care. However, molecular evidence suggests that over 85% of socially monogamous species in fact demonstrate the use of alternative reproductive strategies, including extra-pair mating and conspecific brood parasitism, as part of their behavioral repertoire (Arnold & Owens, 2002; Griffith, Owens, & Thuman, 2002; Owens & Hartley, 1998).

Much research has focused on the mechanisms underlying variation in reproductive strategies across species. However, the extent to which individuals exhibit the ability to flexibly adjust their reproductive strategy in response to ecological and social circumstances is still poorly understood. In particular, what sorts of selection pressures select for plasticity in reproductive strategy? Furthermore, what is the role of experience in allowing breeding females to choose an appropriate reproductive strategy for their ecological and social environment?

Strong selection against high failure rates during the first breeding attempt is thought to select for low variability in reproductive outcomes and, thus, a minimal role for experience. However, it is also possible that organisms in unpredictable environments should in fact be flexible and plastic in their reproductive strategies. Indeed, according to a comparative meta-analysis of many avian species, the incidence of two alternative reproductive strategies, extra-pair paternity and egg dumping, were both strongly linked to fast track reproduction (Arnold & Owens, 2002; Bennett & Owens, 2002). This linkage was found to be especially strong in species with adult mortality rates in excess of 30% and duration of chick feeding of less than 30 days. In addition, there is a positive association between local environmental variability and the use of two alternative reproductive

strategies, extra-pair mating and divorce, in socially monogamous passerines (Botero & Rubenstein, 2012). This suggests that there may, in fact, be greater within-species variability in reproductive strategies in species adapted to unpredictable environments.

Like many passerines, zebra finches (*Taeniopygia guttata*) form long-term socially-monogamous pair bonds and display biparental care. They are always paired, even when not actively breeding (Perfito et al., 2007; Zann, 1994; Zann, 1996). They breed opportunistically in response to unpredictable rainfall and offspring can breed at a very early age (60 days) (Zann, 1996; Zann et al., 1995). In the wild, zebra finches experience very high rates of mortality: in one population the mean annual survivorship for the first 12 months of life was only 4% (Zann, 1996). The average lifespan of wild zebra finches in two populations was found to range from 53 to 128 days (Zann & Runciman, 1994), though they can live significantly longer in captivity. Given that the median age of reproduction is 90 days, the vast majority of zebra finches may only get a single opportunity to breed, if any. However, there are many caveats to this data due to the difficulty in tracking the highly mobile populations. It is plausible that, if an individual survives to adulthood, it may live for several years and have many breeding opportunities. Nevertheless, zebra finches have a high rate of clutch failure, both in natural and captive populations (Zann, 1996; Fenske & Burley, 1995; Millam et al., 2001).

Zebra finches have been found to use alternative reproductive strategies in both field and captive populations, with drastic variation in the rates between different environments. The rate of extra-pair paternity (EPP) is very low in two different field populations (2.4% and 1.7%), but it has been found to be far higher in lab populations (28%, 29%, 15.3%) (Birkhead et al., 1990; Burley et al., 1996; Forstmeier et al., 2011; Griffith et al., 2010; Tschirren et al., 2012). Conspecific brood parasitism (CBP) shows the opposite pattern, with the rate of CBP in the field (10.9% and

5.4%) being somewhat higher than what has been observed in the lab (3.6% and 5.4%) (Birkhead et al., 1990; Burley et al., 1996; Griffith et al., 2010; Schielzeth & Bolund, 2010). Although there is some evidence that individual females may vary in their propensity to engage in extra-pair mating (Forstmeier, 2007), this variability across the lab and field suggests that it is also possible to have plasticity across contexts in the expression of alternative reproductive strategies. Indeed, wild-caught birds breeding in captivity have been found to have a higher rate of extra-pair fertilizations than birds from the same population breeding in the wild (12% versus 1.7%) (Tschirren et al., 2012; Griffith et al., 2010), suggesting that the frequency of alternative reproductive strategies is sensitive to the breeding environment.

Despite their high rates of mortality and rapid development, zebra finches do not fit the classic model of a fast life history species. As opportunistic breeders, they exhibit many adaptations to allow them to survive in a highly unpredictable environment, including timing the onset of breeding to variable environmental cues. They also exhibit permanent rather than short term pair bonds, despite being relatively short lived (Adkins-Regan & Tomaszycki, 2007). Additionally, they show extensive learning in their general social development, including complex socially-guided vocal learning and sexual imprinting (Eales, 1985; Jones et al., 1996; Slater et al., 1988; ten Cate & Vos, 1999; Vos, 1995; Vos et al., 1993; Witte & Caspers, 2006).

Thus, what would be the predicted role for experience and reproductive strategies in a bird with this life history? It has been hypothesized that longer developmental periods correspond to increased learning capacities and the development of complex skills in birds, as well as humans (Heinsohn, 1991; Kaplan et al., 2000; Lack, 1954; Locke & Bogin, 2006). From this perspective, in rapidly-developing species such as the zebra finch, experience should play a minimal role in

reproductive outcomes. However, based on the importance of learning in the general ecology of the zebra finch, we hypothesize that experience plays an important role in breeding, as well.

We performed an in-depth study of reproductive outcomes in a captive population of zebra finches, testing the impact of age and prior breeding experience of females on the use of alternative reproductive strategies and reproductive success. We focused on the role of females, since females are thought to have a stronger influence on reproductive outcomes (Williams, 2012). We recorded data on pairing, egg laying, chick rearing, and fledging success for 112 inexperienced male and 112 female zebra finches. Sixty females had prior breeding experience and 52 females were inexperienced. To test the impact of the social environment on reproductive outcomes, females were randomly assigned to aviaries in which 1) all of the females were experienced, 2) all were inexperienced or 3) half of the females were experienced and half were inexperienced. To measure the use of alternative reproductive strategies, parents and offspring, including eggs that failed to hatch, were genotyped to determine genetic parentage, which was compared to observational data to identify incidences of extra-pair fertilization (EPF) and conspecific brood parasitism. We predicted that females with prior breeding experience would be more skilled parents and more likely to strategically use extra-pair mating to improve their reproductive success.

The role of experience in shaping reproductive strategies and breeding outcomes is still poorly understood. Across many species of birds, there is a strong statistical association between age and breeding success (Forslund & Part, 1995; Fowler, 1995; Lack, 1973; Saether, 1990). One plausible explanation is that older birds may have developed certain skills important either in survival and self-maintenance more generally (such as foraging ability) or in skills specifically related to breeding (such as incubation, chick rearing, or nest defense) (Forslund & Part, 1995; Saether, 1990). However, in natural populations, age is almost always correlated with experience, making

it difficult to disentangle general age effects from learning or from some physiological change from past breeding.

Although there is very little overlap in age between experienced and inexperienced females in our sample, the present study represents an improvement over many previous investigations of the effect of age on breeding success for several reasons. First, we are able to control environmental variability by using a captive population in naturalistic social aviaries. Second, we are able to control, to a limited extent, the contributions of the pair partner, since both temporal constraints related to pair formation and breeding experience of the partner may impact the reproductive success of the pair. Finally, we are able to test the impact of variation in the social environment by experimentally manipulating the breeding experience of the other birds in the same social group.

Methods:

Subjects

The male and female birds used as parents in this study were drawn from several different populations within two domesticated aviary colonies at Cornell University. Birds from these populations varied on several dimensions, including age (and whether information was available about their age) and whether or not information was known about their parentage. To control the composition of birds within breeding aviaries, birds from different populations were divided into blocks and then randomly assigned to aviaries. If information was known about the parentage of birds, possible siblings were randomly assigned to different treatment aviaries. The diet throughout was *ad libitum* Kaytee Forti-Diet Pro-Health Finch feed, grit, cuttle bone, and water.

Females ranged in age from approximately 62 days to 421 days, with an average age of 205 ± 132 days. All males in the study were inexperienced breeders and had been housed in single-sex

aviaries since 40-50 days old. Males ranged in age from 60 days to 562 days old at the start of the study, with the average age of 219 ± 180 days.

Exact hatch dates were known for 71/112 (63.4%) of the females and 97/112 (86.6%) of the males; recording hatch dates was not standard practice in the facility prior to the start of the study. However, fledglings are removed from their natal aviary at around 40-50 days of age, when sexually dimorphic plumage first becomes apparent. At this time, they received a numerical metal band (except one male with a known hatch date). Bands are assigned to individuals in numerical order, meaning that there is a strong correlation between ID number and age ($F_{1,166} = 53.31$, $p < 0.0001$, $r = 0.49$). Thus, we used numerical ID to predict age, when the exact age was not known.

All animal procedures conformed to Federal and State regulations and were approved by the Cornell University Institutional Animal Care and Use Committee (Protocols 2007-0074 and 2008-0001).

Experimental Setup

Within two weeks prior to the start of the study, the mass, tarsus lengths (in triplicate), and a tissue sample (either blood or pin feather) was collected from all parents in the study. Masses were regressed on tarsus length within each sex to produce a mass-tarsus residual score, a measure of body condition. Previously-paired females were visually- and acoustically-isolated from all males for one month prior to the start of the study, presumably including all former partners, to facilitate rapid pairing with a new male.

Females were then randomly assigned to an aviary in one of three treatments: an aviary in which all females had prior breeding experience (all-experienced, $n = 5$ aviaries), all females were inexperienced (all-inexperienced, $n = 4$ aviaries), or an aviary in which there was a 50:50 mix of

experienced and inexperienced breeders (mixed, $n = 5$ aviaries). This created four aviary treatment types: experienced females in all-experienced aviaries, inexperienced females in all-inexperienced aviaries, experienced females in mixed aviaries (mixed-experienced), and inexperienced females in mixed aviaries (mixed-inexperienced). Thus, there were a total of 60 experienced females, 52 inexperienced females, and 112 inexperienced males used as parents in the study. Experienced females had previously been housed in a breeding aviary with males and had the opportunity to pair and breed, but details of their breeding outcomes were not known.

At the start of the study, sixteen adults (8 females, 8 unfamiliar males) were released into one of 14 breeding aviaries, each equipped with eight nest boxes and coconut husk (for nest building). The birds were given the opportunity to pair and breed for a total of 60 days. The first 35 days of the study is referred to as Phase One. Thirty-five days is the minimum length of time in which a pair can pair start a clutch, fledge chicks, and initiate a second clutch, and thus the period in which breeding experience treatment could be guaranteed for the full aviary. All eggs laid between 35 and 60 days were considered to be a part of Phase Two. In Phase Two, all eggs were removed from the nests on the day of hatching and artificially incubated. Eggs that had been laid during the first 35 days but had not yet hatched were allowed to remain in the nest and develop normally. In Phase Two, tissue samples were collected from eggs after 5-8 days of artificial incubation for genotyping. Phase Two provided an opportunity to measure the use of alternative reproductive strategies as a function of reproductive outcomes in Phase One, without the substantial loss of samples due to egg mortality.

Nest Checks

Throughout the study (May 25, 2010 to July 31st, 2010), nests were checked daily between 9am and 11am. The presence of eggs, hatchlings and fledglings was recorded. We also recorded egg condition: buried or not; cracked, missing, or discolored. Eggs were marked on the day laid with pencil. Chicks were marked on the limbs and posterior down feathers with colored permanent marking pens and color-banded at approximately 12 days of age. Previous research in our lab suggests that this does not affect hatchling mortality or parental acceptance. The mass of all chicks was recorded each day until fledging. Any eggs or chicks found dead outside of the nest were collected and, if the identity of the egg could be determined, were also genotyped. The final status of all eggs laid during the study was recorded (broken, buried, egg found outside nest or missing altogether, failed to hatch, nestling death, fledged, and broken in handling).

Observations

There were two types of observations performed. Each morning between the hours of 7am (lights on) and 9am, random focal observations were performed for periods of 5 minutes. This is a period of high activity in the zebra finches and it also the time during which females are predicted to be laying (Slater, 1974). There were 1050 individual observation periods, amounting to 89 hours of observation. Each bird was observed an average 4.76 ± 1.37 times. The bird's location in the aviary as well as the infrequent incidents of aggressive behaviors (attack, chase, beak fence, jabbing, supplant, threat call) were recorded in JWatcher v. 1.0 by observers sitting in front of the aviary. Between the hours of 12-4pm, observations were performed to determine pairing status and nest box occupation, based on clumping, allopreening and two birds occupying the nest box together. These observations were performed throughout pairing, egg laying and chick rearing,

until pairing status was confirmed by multiple independent observers across several observational periods. After pairing status was determined, the proportion of time spent in the nest box, on the perch of the nest box, and on the floor of the aviary was tabulated for the period after the first egg was laid.

Genotyping Procedure

To determine genetic parentage, all parents and offspring were genotyped at six highly-polymorphic microsatellite loci selected from(Forstmeier et al., 2007), based on non-overlapping size ranges, the absence of null alleles, and similar PCR programs. Three primers were labeled in using 6FAM fluorescent tags (*Tgu12*, *Tgu9*, and *Tgu1*) and three were labeled using NED fluorescent tags (*Tgu4*, *Tgu3*, and *Tgu8*).

Genomic DNA was extracted and purified using either the Qiagen DNEasy Blood and Tissue kit (blood and pin feather samples) or Agencourt DNAdvance kit (pin feather and nestling egg/tissue samples). PCR amplifications were performed using the QIAGEN Type-It Microsatellite Kit (QIAGEN, Cat. No. 206243) to perform a multiplex PCR reaction containing all six primer pairs. Each 25-µL PCR contained 12.5-µL of the 2x Type-it Multiplex PCR Master Mix, 2.5-µL 10x primer mix (containing 2µM each primer), 2-µL template DNA, and 8-µL ddH2O. The following is the PCR program used: an initial hot-start 5-min denaturation step at 95ºC; followed by 5 cycle touchdown: 94ºC for 30 s, 60-56ºC for 90 s (dropping 1ºC per cycle), 72ºC for 30 s; another 23 cycles 94ºC for 30 s, 56ºC for 90 s, 72ºC for 30 s; and a final extension at 60ºC for 30 min. PCR products were analyzed on an Applied BioSystems 3730xl Genetic Analyzer. Raw data were analyzed using Genemapper 4.0 software.

Parentage analysis was performed in Cervus, which assigns the most likely candidate parent of each sex from the known parent genotypes within each aviary (Kalinowski et al., 2007). A total of 1368 eggs were laid during the two studies, 639 in Phase One and 729 in Phase Two. Genotypes could be established for 938 (68.6%) samples (see Table 1).

In Phase One, genotypes could be assigned for 310 out of 628 eggs (48.4%). Genetic parentage could be established with 95% confidence for 281 samples. This could be compared to observational data about the expected parents for 272 samples. Following Schielzeth and Bolund (2010), we defined two main categories of eggs: eggs that were laid in the female's own nest and incubated by her or her social partner ('own nest') and eggs that were laid in another nest and not incubated by the female or her partner ('other nest') (Schielzeth & Bolund, 2010). There were only 15 cases of eggs found in an "other nest." Four cases appeared to be linked to dispute over nest ownership and in one case the egg was laid in an inactive nest. Thus, there were only 10 clear cases of conspecific brood parasitism (CBP). Five of those cases were found to be quasi-brood parasitism, in which the observed male owner was the genetic parent, but not the observed female. There were 32 cases of extra-pair paternity out of 272 eggs (11.7% rate), not including the cases of quasi-brood parasitism.

In Phase Two, genotypes could be assigned for 628 out of 729 eggs (86.1%). Genetic parentage could be established with 95% confidence for 568 individual eggs laid. Observed parentage could be compared to the predicted parentage for 525 individuals. In total, 100 eggs were found to have been laid in an "other nest", though 9 of those cases were linked to dispute over nest ownership. This category of "other nest" cannot be subdivided in Phase Two because the eggs were removed on the date they were laid and were not replaced with dummy eggs. Thus, they could not be incubated by the nest owner, so it is not clear whether these 'other' eggs represents CBP attempts,

'egg dumping', or, more likely, the start of a new nest. In addition, there were 49 cases of EPP out of 525 eggs (9.3%).

Heterozygosity

We used the microsatellite genotypes to generate a measure of heterozygosity, multilocus heterozygosity (MLH), calculated as the proportion of heterozygous loci within an individual (Coltman & Slate, 2003; Coulson et al., 1998; Smith et al., 2005). An arcsine transformation was used to analyze MLH data.

Statistical Analyses

All statistical analyses were performed in R v. 2.1.5.1. Non-parametric statistics (Kruskal-Wallis tests or Spearman's ρ) were used to analyze egg and chick count data, which were not normally distributed and could not be modeled using other parametric statistics. Several variables, such as pair formation or whether an egg was a result of a within- or extra-pair fertilization, were coded as nominal data and were analyzed using a generalized linear mixed model with a binomial link function (GLMM; *glmer* in R package *lme4*) with female parent population (cohort) included as a random factor. To analyze the relationship between the probability that a given egg hatched or fledged, we used a GLMM with a binomial link function with female parent identity included as a random factor. Mass-tarsus residuals, age, days to clutch initiation, proportion of time spent in nest box, and arcsine MLH were all introduced as continuous variables. To analyze the relationship between days to first egg and experience, we used a linear mixed model with both aviary and room included as random factors (LMM; *lmer* in R package *lme4*). To test whether EPF eggs differed in heterozygosity, we used a LMM with the genetic female parent identity included

as a random factor. When performing LMM or GLMM, we used a likelihood ratio test (LRT) to compare the full model to a reduced null model with only the factor of interest removed to test for significance of the fixed effect.

Results:

Pair Formation

Only 55 out of 112 females (49%) formed clear pairs with males during the first 35 days (two females formed a same-sex pair). Neither age nor experience predicted whether or not females formed a clear pair (age: GLMM with binomial errors, $\chi^2(1) = 0.00055$, $p = 0.98$; experience: GLMM with binomial errors, $\chi^2(1) = 0.031$, $p = 0.86$). However, female mass-tarsus residual, a measure of body condition, was a predictor of pair formation (GLMM with binomial errors, $\chi^2 = 3.88$, $p = 0.049$; Fig. 1). Although there was a strong correlation both between female mass-tarsus residual and age ($F_{1,110} = 23.31$, $p < 0.0001$, $r = 0.42$) and female mass-tarsus residual and breeding experience ($F_{1,110} = 27.31$, $p < 0.0001$, $r = 0.45$) in our sample, only mass-tarsus residual remained a significant when compared to a reduced model including all other predictors (GLMM with binomial errors with age, breeding experience and mass-tarsus residual as predictors and parent cohort included as a random effect, LRT for mass-tarsus residual: $\chi^2(1) = 5.25$, $p = 0.022$, LRT for age: $\chi^2(1) = 0.0034$, $p = 0.95$, LRT for experience: $\chi^2(1) = 0.55$, $p = 0.46$).

There was a positive correlation between a female's age in days and her partner's age ($F_{1,53} = 6.76$, $p = 0.012$, $r = 0.33$). However, there was no correlation between a female's mass-tarsus residual and the mass-tarsus residual of her male partner ($F_{1,53} = 0.52$, $p = 0.48$).

Reproductive Success

A total of 638 eggs were laid during the 35 day period of Phase One. A detailed breakdown of egg outcomes and whether or not eggs were successfully genotyped can be found in Table 1. A total of 191 eggs hatched, but 73 of those chicks (38%) died prior to fledging. Thus, only 118 chicks survived until fledging—a fledging success rate of only 18.8%. However, the success rate was higher for the eggs that remained in the nest (118 out of 240 eggs, 49.2%). Of the 729 eggs laid in Phase Two (see Table 1), 545 (74.7%) appeared to develop normally in the artificial incubator. The remainder did not develop normally and were either unfertilized or the embryo died within the first few days of development (no data was collected for 9 eggs). Of the 175 eggs that failed to develop, 92 (52.6%) were successfully genotyped.

Given that a female paired, none of the measures were significant predictors of egg or chick numbers in Phase One. Female breeding experience did not predict the number of hatched or fledged chicks in the nest (hatched: Kruskal-Wallis test, $\chi^2 = 0.17$, $p = 0.68$; fledged: Kruskal-Wallis, $\chi^2 = 0.69$, $p = 0.41$). Female age did not predict the number of hatched or fledged chicks (hatched: Spearman $\rho = 0.071$, $p = 0.60$; fledged: Spearman $\rho = 0.14$, $p = 0.31$). Female mass-tarsus residual not a significant predictor of the number of hatched chicks in the nest (Spearman $\rho = 0.004$, $p = 0.98$) or the number of fledged chicks (Spearman $\rho = 0.094$, $p = 0.49$).

However, there was a significant difference in the hatching success of the eggs of experienced and inexperienced females, controlling for female as a random factor (GLMM with binomial errors, $\chi^2(1) = 10.89$, $p = 0.00097$). Inexperienced females in fact hatched a higher percentage of eggs than experienced females (38.3% versus 30.5%), though they laid fewer eggs overall (217 versus 338). Additionally, there was a non-significant trend suggesting that an egg hatched in the nest of an experienced female was more likely to fledge, again controlling for female as a random

factor (GLMM with binomial errors, $\chi^2(1) = 2.95$, $p = 0.086$). A larger proportion of the eggs that hatched in the nest of experienced females fledged in comparison to eggs in the nest of inexperienced females (71.7% versus 57.3%).

Females with prior breeding experience initiated their clutch (laid the first egg in nest) on average 2.3 days faster than inexperienced females (LMM, $\chi^2(1)=10.82$, p = 0.0010) with both aviary and room included as random effects. This relationship remains significant when controlling for mass-tarsus residuals (LMM, $\chi^2(1) = 9.65$, $p = 0.0019$). There was a strong negative association between age and the days to clutch initiation (LMM, $\chi^2(1) = 11.80$, p = 0.00059), including aviary and rooms as a random factor (Fig. 2). Furthermore, this association exists even when very young birds (younger than 90 days) are excluded from the analysis (LMM, $\chi^2(1) = 9.21$, p = 0.0024). Thus, this result is not driven by presence of very young birds in the data set.

The aviary treatment type (all-experienced, all-inexperienced, mixed-inexperienced, mixed-experienced) did not impact the number of hatched or fledged chicks, given that a female had paired (Kruskal-Wallis, hatched: $\chi^2(3)= 1.40$, $p = 0.70$; fledged: $\chi^2(3) = 1.38$, $p = 0.71$). However, there was a significant interaction between aviary treatment type and experience in the fledging success of hatched eggs, controlling for both aviary and room as random factors (GLMM with binomial errors, $\chi^2(1) = 5.24$, $p = 0.022$) (Fig. 3A). Eggs hatched in the nests of experienced females in all-experienced aviaries were more likely to fledge than eggs hatched in the nests of experienced females in mixed aviaries. In contrast, eggs hatched in the nests of inexperienced females in mixed aviaries were more likely to fledge than eggs hatched in the nests of inexperienced females in aviaries in which all the females were inexperienced.

Observational Data

In females who paired, aviary treatment type was a significant predictor of the proportion of time a female spent inside the nest box controlling for treatment cage and room as random effects, presumably incubating their eggs or brooding chicks (LMM, $\chi^2(1) = 4.55$, $p = 0.033$, Fig. 3B). Inexperienced females in all-inexperienced aviaries spent less time in their nest box than all other treatment groups, whereas experienced females in all experienced aviaries spent significantly more time in their nest box. No other behavioral measure was significantly different among aviary treatment types or associated with any other measures.

Alternative Reproductive Strategies

We found a very small number of cases of conspecific brood parasitism in Phase One (10 out of 272 eggs, 3.7%) and all of these were from only two females. Because the number of CBP cases was so small, it is not possible to test hypotheses about what influenced the rate of CBP. However, there was a much higher rate of extra-pair fertilizations (EPF): 32 out of 272 eggs (11.7%) in Phase One and 49 out of 525 eggs (9.3%) in Phase Two were from EPFs. This was a fairly common reproductive strategy, as 15 out of the 73 females who laid eggs (20%) laid at least one egg fertilized by an extra-pair male in Phase One.

Predictors of EPFs

Female breeding experience, female age, female body condition and male body condition were not significant predictors of whether a female laid an EPF egg in Phase One. Thus, we tested several hypotheses related to offspring quality which may indicate that females obtain genetic benefits from extra-pair eggs. The following analyses were performed using generalized linear

mixed effects models, including whether egg was an EPF egg as a fixed effect and female parent identity as a random effect. Extra-pair chicks did not appear to be better on the measures of quality that we collected. Extra-pair eggs were no more likely than within-pair eggs to hatch (GLMM with binomial errors, $\chi^2(1) = 1.28$, $p = 0.26$) or fledge (GLMM with binomial errors, $\chi^2(1) = 0.51$, $p = 0.47$). EPF chicks did not weigh more at fledging (LMM, $\chi^2(1) = 1.57$, $p = 0.21$). Extra-pair eggs were also no more likely to develop normally when artificially incubated (GLMM with binomial errors, $\chi^2(1) = 1.02$, $p = 0.31$), a stronger test of the indirect benefits hypothesis of extra-pair mating.

However, extra-pair offspring may benefit from increased genetic variability, consistent with the heterozygosity theory of mate choice (Brown, 1997). There was a non-significant trend suggesting that extra-pair eggs were more heterozygous than within-pair eggs using the arcsine-transformed multilocus heterozygosity (MLH) and controlling for female parent identity as a random effect (LMM, $\chi^2(1) = 3.42$, $p = 0.064$) (Coulson et al., 1998). However, heterozygosity was not a significant predictor of hatching (LMM, $\chi^2(1) = 0.91$, $p = 0.35$) or fledging success (LMM. $\chi^2(1) = 0.12$, $p = 0.73$).

In Phase Two, the EPF rate among females who had a failed breeding attempt in Phase One was 39 out of 243 eggs (16%) versus 10 out of 144 eggs (6.9%) among females that had been successful. There was a nearly significant negative association between the probability that a Phase Two egg was fertilized by an extra-pair male and the number of fledged chicks in the female's nest in Phase One, controlling for female as a random factor (GLMM with binomial errors, $\chi^2(1) = 3.73$, $p = 0.054$). There were no clear trends indicating which causes of nest failure (aborted nests, hatching failure or nestling deaths) may have led females to pursue extra-pair matings in our data.

Discussion:

Reproductive Outcomes

Consistent with previous studies in zebra finches, we find that even in a captive colony with minimal foraging demands, there is a great deal of variability in breeding success (Millam et al., 2001). This degree of variability is especially remarkable in a species with high-mortality and fast development, where there is expected to be very strong selection against high failure rate in reproduction.

The observed rate of pair formation was low, though not markedly different from that found in previous studies in our lab using similar numbers of birds in pairing aviaries (Adkins-Regan & Tomaszycki, 2007). In a study in which behavioral evidence of pair formation between two males and two females was measured, the failure to form a clear pair bond was associated with the two males courting one female in preference over the other (Silcox & Evans, 1982). In this context, the finding that female mass-tarsus residual is the only predictor of pair formation perhaps suggests that males prefer to court and pair with females in better body condition, leaving females in poor condition without a partner (Jones et al., 2001).

We also find evidence that, within pairs, zebra finches appear to mate assortatively by age. Assortative mating by age has been found in a number of avian species (Coulson & Thomas, 1983; Lessells & Krebs, 1989; Nisbet et al., 1984), though it has not previously been observed in zebra finches, to our knowledge. Often this assortative mating is attributable to structural factors, such as younger individuals arriving at the breeding grounds later in the season or forming pairs with individuals who reach reproductive maturity around the same time. Since the timing of the initiation of pairing was controlled, no such temporal factors can explain the assortative mating observed in our study. Furthermore, the lack of correlation between body condition of male and

female partners suggests that zebra finches may be using alternative cues for age rather than condition to pair assortatively.

Despite the variability in reproductive outcomes, there are few reliable predictors of reproductive success. However, we find several ways in which breeding experience and age each appear to provide significant reproductive benefits. First, inexperienced/younger females lay fewer eggs (possibly due to the later initiation of breeding), but have a slightly higher hatching success relative to experienced/older females. However, there is a non-significant trend suggesting that older females with prior breeding experience are more likely to be successful in raising hatched chicks to survival. Additionally, older/experienced females are significantly more successful at raising chicks to fledging when housed in aviaries with other experienced females. These results suggest that the greatest benefit of age and experience may come during the chick rearing phase, rather than during incubation, where particular skills, such as feeding or brooding, improve with experience. However, this trend requires further exploration.

Older/experienced females also initiate clutches sooner. This result suggests that either age or experience prime the female to produce eggs more quickly. It is also possible that older or experienced females progress more quickly through the courtship, pairing and nest-building phases of breeding, despite the inexperience of their partners. In a previous study, individuals breeding a second time with a previous partner also initiated clutches approximately three days faster than birds who were experimentally forced to re-pair (Adkins-Regan & Tomaszycki, 2007). Thus, it remains to be tested whether the observed acceleration in egg laying is a function of behavioral or physiological readiness of the female. Nevertheless, given the importance of rapid reproduction in opportunistic breeders such as the zebra finch, faster clutch initiation is likely to be an ecologically-relevant benefit.

Finally, the impact of the breeding experience of other females within the aviary (aviary treatment type) provides suggestive evidence that social interactions between experienced and inexperienced females may impact reproductive outcomes for both. Given that females were randomly assigned to all-same or mixed aviaries, any differences between groups can be attributable to social factors. Inexperienced females, in particular, seem to benefit from sharing an aviary with experienced females, substantially increasing their fledging success. Inexperienced females in mixed aviaries are indistinguishable from experienced breeders in the amount of time spent inside their nest box. This suggests that they may spend more time incubating when in a social group with more experienced females. Experienced females, however, appear to do worse when in an aviary with inexperienced females. It is unclear what particular changes in the social environment may have led to these different outcomes, but an intriguing possibility that inexperienced breeders may learn how to be better parents by observing conspecifics. Another possibility, based on the differential-allocation hypothesis, is that females vary their parental investment in response to their relative desirability as mates (Burley, 1986, 1988). If they are in a social group in which all females are older, more experienced and in better body condition, they may increase their parental investment in order to more effectively compete for mates, whereas females in social groups with more variability do not need to differentially allocate parental investment. These hypotheses remain to be more fully tested, however.

Extra-pair Paternity

The rate of extra-pair paternity in this study (81 out of 797 total eggs, 10.2%) is consistent with, though on the low end of, other studies in captive populations of zebra finches (Birkhead et al., 1990; Burley et al., 1996; Forstmeier et al., 2011; Griffith et al., 2010; Schielzeth & Bolund,

2010). One possible reason for the higher rate in captive populations more generally is that these birds face a substantially different social environment than field populations. These differences may include, but are certainly not limited to, breeding at higher densities than what is found in the field. Additionally, individuals are in constant proximity to the nest, since they cannot leave the nesting site to forage as they would in the wild. Thus, higher rates of extra-pair paternity in the lab are consistent with the observation that EPP occurs at a higher frequency when individuals nest at higher densities (Birkhead & Moller, 1993; Gowaty & Bridges, 1991; Moller & Ninni, 1998; Westneat & Sherman, 1997).

Nevertheless, the plasticity of alternative reproductive strategies, including the significant differences across field and lab populations, suggests a great deal of flexibility in reproductive behaviors. Future research should investigate the specific cues and environmental factors that may underlie the flexible adjustment of reproductive strategies. This is especially important because the zebra finch has become a model organism, commonly studied for its social and reproductive behavior in the lab (Griffith & Buchanan, 2010).

None of the measured female characteristics were associated with an increased rate of extra-pair paternity among females. One reason for this may be that the rate of extra-pair paternity is in fact more related to intrinsic features of the female unrelated to age, breeding experience or body condition (Forstmeier, 2007).

However, we tested several hypotheses related to offspring quality which may indicate that females may obtain genetic benefits from extra-pair eggs. Although extra-pair eggs did not appear to benefit from increased growth or survival before or after hatching, extra-pair offspring may benefit from increased genetic variability, consistent with the heterozygosity theory of mate choice (Brown, 1997). Microsatellite heterozygosity has been found to be correlated with measures of

health and survival in multiple species (see (Hansson & Westerberg, 2002) for review), though the correlations are generally quite weak (Coltman & Slate, 2003). Nevertheless, the trend suggesting that extra-pair offspring are more heterozygous than within-pair offspring suggests either that females are mating with extra-pair males with higher overall heterozygosity (Borgia, 1979; Mitton et al., 1993) or that females are mating selectively with extra-pair males who are more dissimilar from themselves than their social mate (Houtman, 1992). Another intriguing possibility is that females mate multiply and use a 'genetically loaded raffle' to ensure that their extra-pair offspring benefit from increased heterozygosity (Griffith & Immler, 2009).

Furthermore, unlike a recent experimental study, we find evidence suggesting that female zebra finches who failed to successfully fledge a chick in Phase One laid a higher proportion of EPF eggs in a second breeding attempt, although the trend did not reach significance (Ihle et al., 2013). There are several possible reasons this outcome may differ from previous findings. Our measure of reproductive failure was the failure to successfully raise at least one chick to fledging during the first breeding attempt, as opposed to hatching success. Failure to fledge any chicks may be a more robust predictor of switches in reproductive strategies than hatching failure. Second, because we did not experimentally manipulate nesting failure, females in this study had the benefit of the full complement of natural cues related to her male partner and his quality that may have directly resulted in the failed breeding attempt. This finding that experience from one breeding attempt may influence behavior during the second attempt leaves open the possibility that learning influences reproductive investment and the flexible use of alternative reproductive strategies in zebra finches.

However, given the observational nature of this study, another plausible explanation is that females who mated multiply during Phase One were also more likely to have lower hatching

success, perhaps due to less help from the pair partner. As a result, we may have obtained a biased sample of eggs from promiscuous females during Phase One, leading to the association between fledging failure in Phase One and promiscuity in Phase Two. Because of the sampling method, we do not have the ability to disentangle these two explanations with the current data.

There are several limitations to this study. First, because there is very little overlap in age between experienced and inexperienced females in our sample, we are not able to disentangle the effects of age and experience. A fully experimental design, in which age is not a confound, is still needed to confirm these tentative findings. Second, we performed many tests on this data set, but we chose not to correct for multiple testing. Some findings would not remain significant if we chose a p-value of less than 0.05, but because this was an exploratory study we have presented these findings with an uncorrected p-value. Finally, this work was performed on a population of domesticated zebra finches, whose life history differs from wild populations in significant ways. Clearly, the ecological and social environment in which the birds are breeding can have a significant impact on reproductive outcomes, which means that extrapolation from lab populations to the field is risky. Nevertheless, these findings provide evidence consistent with previous work in zebra finches and suggest several avenues for future research on the role of experience in breeding outcomes.

Conclusions:

Consistent with recent evidence and theories regarding the evolution of flexible mating strategies, we find evidence that age and breeding experience impact reproductive outcomes in the zebra finch. Older and experienced females initiate egg laying faster and appear to be more successful at rearing chicks until fledging, though inexperienced females have slightly better

hatching success. The social environment matters as well, with the breeding experience of other birds within the same social group influencing reproductive outcomes. Females also appear to use information about the success of one breeding attempt to make decisions about a second attempt, potentially switching strategies and pursuing adaptive extra-pair mating when the first attempt was unsuccessful. Future research should investigate the specific mechanisms by which experience influences reproductive outcomes, particularly to test whether the improvement in reproductive success is a result of learning, physiological changes or some other mechanism.

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Figures

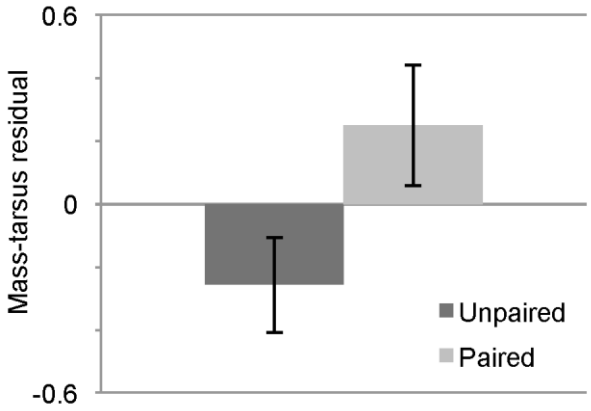

Figure 1: Female pairing status by body condition

Mean female mass-tarsus residual ±SE, a measure of body condition, for 112 females, depending on whether or not they formed a clear pair with a male.

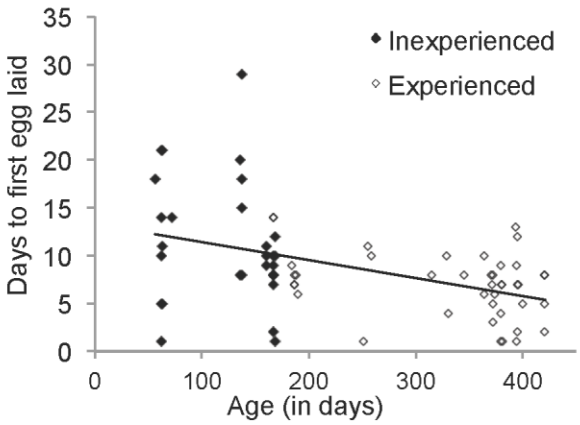

Figure 2: Days to clutch initiation by age and breeding experience

Each data point represents a single female (N = 71) that laid an egg during Phase One. Females with no prior breeding experience are shown with the closed symbols and females with breeding experience are shown with open symbols. The line depicts the fitted linear regression; the analysis used to test for significance was a linear mixed model controlling for aviary and room as random factors.

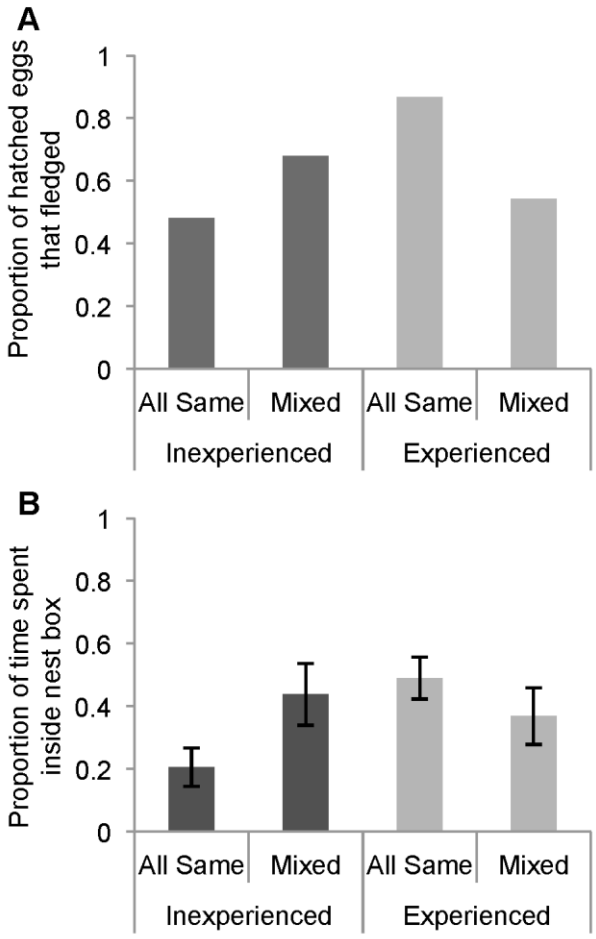

Figure 3: Hatched eggs and proportion of time in nest box by treatment type

Relationship between aviary treatment type and A) the proportion of the hatched eggs that fledged for females in each of the four aviary treatment types (N = 174 hatched eggs with a known female parent) and B) the mean ±SE proportion of time females spent inside the nest box after the first egg was laid (N = 49 females assigned to a nest box). There are four aviary treatment types: inexperienced females in all-inexperienced aviaries (All Same-Inexperienced), inexperienced females in mixed aviaries (Mixed-Inexperienced), experienced females in all-experienced aviaries (All Same-Experienced), and experienced females in mixed aviaries (Mixed-Experienced). Results from inexperienced females are shown in dark grey and experienced females in light grey.

Tables

	Successful Genotyping		***No Attempt/ Failed***	
	Phase One	Phase Two	Phase One	Phase Two
Failed to Hatch	49	-	52	-
Nestling Death	73	-	0	-
Fledged	118	-	0	-
Buried	16	-	102	-
Outside Nest	18	-	95	-
Broken	28	-	45	-
Broken in Handling	8	-	31	-
Incubated - Failed to Develop	-	92	-	83
Incubated - Embryo	-	536	-	9
No sample	-	-	4	9
Total Numbers	*310*	*628*	*329*	*101*
Parentage Assigned w/ 95% confidence	281	568	-	-
Compared to Observed Parentage	272	525	-	-

Table 1: Egg Outcomes and Genotyping Success

Eggs listed under 'Successful Genotyping' were genotyped at a minimum of 5/6 loci. 'No Attempt/Failed' refers to eggs that we were not able to successfully genotype, either due to lack of sample or multiple failed genotyping attempts. In both cases, the number of eggs in each final status category is listed: Failed to Hatch, Nestling Death, Fledged, Buried, Outside Nest, Broken, Broken in Handling and No Sample in Phase One and Incubated – Failed to Develop and Incubated – Embryo and No Sample in Phase Two. For eggs that were successfully genotyped, the number of eggs for which parentage was assigned with 95% confidence and the number of eggs that could be compared to observed parentage are listed.

CHAPTER THREE

Developmental effects of vasotocin and nonapeptide receptors on early social attachment and affiliative behavior in the zebra finch

Abstract:

Zebra finches demonstrate selective affiliation between juvenile offspring and parents, which, like affiliation between pair partners, is characterized by proximity, vocal communication and contact behaviors. This experiment tested the hypothesis that the nonapeptide arginine vasotocin (AVT, avian homologue of vasopressin) and nonapeptide receptors play a role prior to fledging in the development of affiliative behavior. Zebra finch hatchlings of both sexes received daily intracranial injections (posthatch days 2-8) of either AVT, Manning Compound (MC, a potent V1a receptor antagonist) or saline (vehicle control). The social development of both sexes was assessed by measuring responsiveness to isolation from the family and subsequent reunion with the male parent after fledging. In addition, we assessed affiliative preferences for the parents, unfamiliar males, and unfamiliar females each week throughout juvenile development. Compared to controls, MC subjects showed decreased attachment to the parents and MC males did not show the normal increase in affiliative interest in opposite sex individuals as they reached reproductive maturity. In contrast, AVT subjects showed a strong and sustained affiliative interest in parents throughout development, and males showed increased interest in opposite sex conspecifics as they matured. These results provide the first evidence suggesting that AVT and nonapeptide receptors play organizational roles in social development in a bird.

Introduction:

Early in the development of species that exhibit parental care, young offspring often form close social and affiliative relationships with family members—they become attached to their parents and siblings. Attachment is commonly defined as a selective social or emotional bond, measured by maintenance of proximity, voluntary contact, or selective or differential behaviors towards the attachment object, as well as distress when separated from it (Ainsworth, 1989; Carter et al., 1995). Depending on the species, the onset of sexual maturity often coincides with interest in non-family members, especially potential mating partners. In species that exhibit both parental care and pair bonding in adulthood, young seem to transition from an exclusive close relationship with the family to an adult pair relationship similarly characterized by attachment and affiliation.

Zebra finches (*Taeniopygia guttata*) exhibit socially monogamous pair bonds in adulthood and demonstrate a shift in affiliative preferences during juvenile development (Adkins-Regan and Leung, 2006; Immelmann, 1972; Zann, 1996). The young fledge around day 18 post-hatching, but remain dependent on parental feeding until approximately 35 days of age, though they remain in contact with parents until around 48 days of age and sometimes into adulthood (Boogert et al., 2014; Zann, 1996). As the juveniles progress toward reproductive maturity, the objects of their affiliation change from the parents and siblings to potential partners, followed by the formation of permanent pairs.

Zebra finch chicks must demonstrate the motivation to remain proximal to parents and family only after leaving the nest, which requires both the recognition of the parents and selective behaviors directed towards them. In the wild, zebra finch fledglings are left alone for significant amounts of time, though the parents will return at regular intervals from their foraging bouts to feed the fledglings (Zann, 1996). When alone, the young typically remain inconspicuous by

clumping together silently and motionlessly with their siblings (Zann, 1996). However, the fledglings will respond to adult distance calls with their immature vocalization, known as the long tonal call. When their parents arrive, the fledglings are observed to hop towards them, emitting the long tonal call, which often progresses into the begging call (Zann, 1996). Zebra finch fledglings will preferentially respond to the distance calls of their parents, particularly their fathers, though this specificity appears to develop over the course of several days (Mulard et al., 2010). Recognition of the parents by the fledglings is commonly observed in other colonial and nidiculous species, suggesting that this behavior is a widespread phenomenon (swallows (Beecher et al., 1981; Leonard et al., 1997; Medvin and Beecher, 1986; Sieber, 1985; Stoddard and Beecher, 1983), jays (McArthur, 1982) and seabirds (Aubin and Jouventin, 2002; Beer, 1969; Charrier et al., 2001; Evans, 1970; Mulard et al., 2008)).

Despite decades of research on the development of early social attachments, such as classic research on filial imprinting and vocal learning in birds, the development of the neural and neuroendocrine mechanisms mediating the formation and maintenance of selective affiliative relationships is still largely a mystery (Hoffman, 1987; Immelmann, 1975; Lorenz, 1937). Nonapeptides in the oxytocin family (mesotocin (MT) and arginine vasotocin (AVT) in birds; oxytocin (OT) and arginine vasopressin (AVP) in mammals) have been implicated as important modulators of social behaviors, though the vast majority of this research has focused on the activational effects of these peptides in adult animals. Nevertheless, convergent neurochemical, anatomical and behavioral evidence suggests that these nonapeptides acting in the reciprocally-connected network of brain nuclei known as the social behavior network are important in the formation and maintenance of selective affiliative relationships with conspecifics across a wide range of vertebrate species (Goodson, 2005; Newman, 1999; O'Connell and Hofmann, 2011).

The primary sources of nonapeptides that act on receptors within the social behavior network derive from the AVP/OT cell groups of the supraoptic (SON) and paraventricular (PVN) nuclei of the hypothalamus, as well as from smaller extrahypothalamic accessory cell groups, including the medial amygdala (meAMY), medial bed nucleus of the stria terminalis (BSTm), lateral septum (LS), olfactory bulb (OB), and suprachiasmatic nucleus (SCN) (Choleris et al., 2013; Laycock, 2009). Importantly, the distribution of nonapeptides cell bodies and their receptors is species specific (e.g. many mammals, but not birds, exhibit nonapeptides cell bodies in the LS) (Kelly and Goodson, 2014a).

Until very recently, most research on the neural mechanisms of attachment and pair bonding has focused on the socially monogamous prairie vole (*Microtus ochrogaster*) (McGraw and Young, 2010). However, there is increasing evidence that the nonapeptides play an important role in affiliative behaviors in birds. Two recent studies showed that antagonists which act primarily at the VT3 (OT-like) receptor increased the latency to pair and decreased pair formation in zebra finches when administered both centrally and peripherally (Klatt and Goodson, 2013; Pedersen and Tomaszycki, 2012). Additionally, pairing for 48 hours was found to increase expression of both AVT and MT in the PVN in both sexes and AVT in the BSTm in males (Lowrey and Tomaszycki, 2014). Consistent with this finding, antisense knockdown of MT in the PVN significantly increased the latency to pair in females and reduced affiliative behaviors in zebra finches of both sexes (Kelly and Goodson, 2014b). Knockdown of AVT production in the PVN also reduces gregariousness in both sexes (Kelly and Goodson, 2014b). In several species of birds, there is an increase in the expression of c-Fos, an immediate early gene, in AVT-producing neurons in the BSTm in response to positively-valenced social stimuli, including potential mating partners (Goodson et al., 2009; Goodson and Wang, 2006). Males that failed to reliably court females had

fewer AVT neurons in the BSTm than did reliable courters and they failed to show an induction of c-Fos expression in response to exposure to a female conspecific (Goodson et al., 2009). However, partner preference is not induced by central infusions of either AVT or MT in adult zebra finches, suggesting that the prairie vole findings do not generalize to zebra finches, at least in the details (Goodson et al., 2004).

Despite the evidence that the nonapeptides play an important role in mediating social relationships across taxa in adulthood, there is not yet a complete story regarding their role in the development of social behaviors in any species (Cushing, 2013). Some evidence suggests that manipulations of the AVP system during development can profoundly affect social behaviors of both juvenile and adult rats (Boer, 1985; Boer et al., 1994; Bredewold et al., 2014; Schank, 2009; Veenema et al., 2012; Winslow and Insel, 1993). In prairie voles, injections of OT or OT antagonists on postnatal day 1 lead to changes in nonapeptide binding in several brain regions in adults, but the directions of these changes are age-dependent, site-specific and sexually dimorphic (Bales et al., 2007; Yamamoto et al., 2004). Prairie voles of both sexes exposed to OT on postnatal day 1 appeared to show a facilitation of pair formation and partner preference as adults (Bales and Carter, 2003a, 2003b). Neonatal exposure to AVP increased aggression in adult male prairie voles (Stribley and Carter, 1999). Many puzzles remain, however; species differences between prairie and montane voles in the behavioral response to social isolation are present long before differences in the AVP/OT receptor distributions appear (Shapiro and Insel, 1990; Wang and Young, 1997). Nevertheless, this work in rodents has led to widespread speculation that nonapeptides may be implicated in the development of social deficit disorders in humans (Carter, 2007; Insel, 2010; Kenkel et al., 2014; Marazziti and Dell'Osso, 2008).

To our knowledge, there is only one study providing evidence that the nonapeptides underlie

differences in social behaviors during development in birds: systemic injections of AVT altered approach behavior in newly-hatched ducklings (Martin et al., 1979; Martin and Van Wimersma Greidanus, 1978). These findings suggest that AVT may be important during the sensitive period for filial imprinting. The timing of the development of nonapeptide systems as outlined in rodents and the important role that nonapeptides play in adulthood across a wide range of taxa provide convergent evidence for the hypothesis that AVT plays an organizational role in the development of species-typical affiliative behaviors in the zebra finch. Organizational effects of a hormone occur early in development, when they establish the neural and physiological substrate for future behavior (Phoenix et al., 1959). Organizational effects are thought to occur during a critical window or sensitive period in development and exert permanent and long-lasting effects for the life of the individual.

In this experiment, we manipulated the nonapeptide system of zebra finch chicks on days 2-8 posthatching via daily intracranial injections of either AVT, Manning Compound (MC, a potent V1aR antagonist) or saline (vehicle control) and assessed the development of social attachment and affiliative behaviors across juvenile development. We first assessed attachment in a social isolation test the first day after fledging and then in weekly four-way affiliative preference tests from post-hatch day 30 to 86. We hypothesized that AVT and activity of the nonapeptide receptors serve to increase the salience of, attention to, or reward value of interactions with conspecifics, leading to alterations to attachment to the parents, as well as changes in the affiliative preferences for opposite sex individuals as the subjects reached sexual maturity. We predicted that AVT injected birds would show a stronger affiliative preference for the family early in development compared to controls and that this preference would be sustained throughout development. We also predicted that Manning Compound injected birds would not show strong affiliative

preferences for any birds, less affiliation overall, and diminished interest in opposite sex birds.

Materials & Methods:

Breeding Conditions

Seventy-two unpaired adult males and females (hereafter "parents") were assigned to one of six breeding aviaries (1.2 x 0.9 x 0.6 m) and allowed to pair and breed. Offspring hatched within 40 days became the experimental subjects used in the study. Until approximately 40 days of age, subjects were cared for by the parents, which were provided with *ad libitum* access to finch seed, cuttlebone, grit, water, and supplemented weekly with hard-boiled egg. Parent pairs and nest box occupancy were determined based upon the display of pair maintenance behaviors, including clumping, allopreening, and the occupancy of a nest box together. Observations were performed multiple times by independent observers until pairing status was confirmed. Nests were checked daily and the number of eggs, number of chicks, and chick mass was recorded. Chicks were marked using colored non-toxic permanent marking pen, re-applied daily.

Genetic Sexing

In order to balance the sex ratios across treatment, subject chicks were genetically sexed on the day of hatching using DNA extracted from feather follicle tissue. PCR was performed using primers developed by Soderstrom et al. (2007). Each 10µL PCR reaction contained 6.3 µL RNAse-free H_20; 1 µL 10x Rxn Buffer (10x PCR -$MgCl_2$); 0.4 µL 50mM $MgCl_2$; 0.2 µL Deoxynucleotide Solution Mix, 8 µmol each dNTP; 1 µL W+Z Primer Mix containing 2µM of each primer; 0.1 µL Platinum® Taq DNA Polymerase; and 1 µL of sample DNA. The gene fragments were amplified by PCR using the following cycling conditions: an initial hot-start 4-min denaturation step at 95ºC;

followed by 35 cycles of 94ºC for 30 s, 62ºC for 45 s, 72ºC for 45 s; and a final extension at 72ºC for 10 min. Results were visualized on a 1.25% agarose gel.

Intracranial Injections

Starting on Day 2 post-hatching through Day 8, subjects received daily 2µL intracranial (IC) injections of either 1) AVT (10ng, (Arg8)-Vasotocin, Bachem 1785.0005); 2) Manning Compound (MC), a potent V1a and mild OT receptor antagonist (50ng, d(CH2)51,Tyr(Me)2,Arg8)-Vasopressin, Bachem 5350.0005); or 3) 0.9% isotonic saline (Castagna et al., 1998; Goodson et al., 2004; Manning et al., 1989). These dosages were based on those used in intracerebroventricular infusions in adults in Goodson et al., 2004 and scaled by 1/5, based on the changes in brain volume between adults versus juveniles (Ikebuchi et al., 2012). Both AVT and MC are predicted to act at multiple receptor subtypes in the zebra finch brain, including the VT4 (V1aR), VT3 (OT-like), and V2 receptors (Busnelli et al., 2013; Kruszynski et al., 1980; Leung et al., 2009; Manning et al., 2012).

IC injections were performed using a sterile stainless steel insulin syringe (Beckman Dickman, U-100 BD Ultra-Fine Short Lo-Dose™ Insulin Syringes, 31 Gauge, 0.5mL volume, 8mm needle length), similar to Bender & Veney (2008). This technique is feasible because the zebra finch hatchling skull is thin, flexible and easily penetrable by a needle. To perform the IC injection, 2µL was pipetted onto a sterile petri dish using a sterile barrier tip pipette. The bead of liquid was carefully drawn into the tip of the syringe. The needle was then shallowly inserted bevel down at a 45° angle on the top of the cranium at the midline. The chicks continued to behave normally immediately following injections, including normal begging and locomotor behavior. The chicks also gained weight and developed normally (Fig 1a) and there was no increase in mortality associated with the IC injections or any of the treatments.

The efficacy of intracranial injections was verified by injecting two non-subject chicks (Day 2 and Day 8) with 2µL of India ink, diluted 1:10 in 0.9% saline. After four hours, the chicks were euthanized by isoflurane overdose, decapitated, and the whole head was flash frozen and sectioned to determine where in the brain India ink was present (see Supplementary Fig. 1). In both the Day 2 and Day 8 chicks, India ink was found primarily along the lateral ventricles. There was also more limited staining within the hypothalamus along the third ventricle in the Day 8 chick. All procedures were developed with veterinary supervision and approved by Cornell University's Institutional Animal Care and Use Committee.

Chicks of each sex were randomly assigned to a treatment group on day two, following genetic sexing. Chicks within the same clutch were randomly assigned to different treatment groups, such that treatment was unrelated to hatching order. The number of birds that completed treatment and survived until fledging are as follows: AVT Males (N = 11); MC Males (N = 11); Control males (N = 8); AVT Females (N = 9); MC Females (N = 10); and Control Females (N = 11). One control male was found dead on post-hatch day 38 and one control female was euthanized on post-hatching day 51 due to a leg injury.

Zebra finches typically become independent of parental feeding at 40 days of age and reach sexual maturity between 60 and 90 days of age (Zann, 1996). Thus, after approximately 40 days of age (39.8±5.4 days), subjects were removed from their natal aviary and housed in same-sex aviaries in a separate room from the parents. Each same-sex aviary contained birds of the same treatment to control for possible social interactions between birds in different treatments. For a timeline of the experiment, see Fig. 1b.

Social Isolation Tests

The day after subjects were first observed having fledged from the nest, we assessed subjects' responses to social isolation and subsequent reunion with the male parent. In the wild, male parents take a more active role in caring for the fledglings, because the female parent is more involved in incubation if the pair starts a second clutch (Zann, 1996). The social isolation tests (SI tests) were performed in a testing apparatus (60 x 41 x 36 cm) in a separate room from the breeding cage. Two aviaries of paired adults were in the room but were behind a curtain to provide ambient colony noise. A nest box was attached to both the right and left sides of the testing apparatus. The test nest boxes were filled with coconut fibers, imitating the structure of a nest. After one minute of acclimation, we recorded behavior in isolation for nine minutes total. Next, the male parent was placed in the aviary with the fledgling for five additional minutes. The video was scored for the number of perch hops, saccadic head movements, and long tonal calls performed by the subject per minute.

Four-way tests

Preferences for conspecifics were assessed weekly from day 30 to 86 in four-choice proximity tests with two males, two females, the parent pair, or no conspecifics as the four stimulus choices, similar to Adkins-Regan & Leung (2006). Proximity is a valid indicator of family and sexual and pairing interest in this species, because these relationships are marked by close physical proximity (Clayton, 1990). The testing period, with nine weekly tests, covered the majority of the juvenile period, allowing us to measure changes in affiliative preferences across juvenile development.

For testing, the subject was removed from its aviary and placed alone in a testing cage (61 x 61 x 41 cm) in a separate testing room, which was flanked on three sides by cages containing pairs

of stimulus birds. The three stimulus cages (61 x 36 x 45 cm) were positioned next to the subject's cage. One stimulus cage contained the subject's parents, one contained two unfamiliar adult females, and one contained two unfamiliar adult males. The stimuli were placed first into the apparatus and allowed to acclimate for at least one minute prior to the introduction of the subject. Subjects were allowed to acclimate in the apparatus for one minute prior to recording. Tests were 15 minutes long and were videotaped from behind a blind with no human in the room. The testing cage contained three stimulus proximity zones, delineated by perches and tape on the floor. Each corner of the stimulus cage was blocked by wire mesh, creating a cross-shaped testing apparatus, with a perch near each of the four sides of the cage. The remainder of the cage (the center portion and the zone nearest to the video camera) was considered a neutral (non-proximity) zone. The same pool of 20 males and 20 females was used as stimuli in a random order for each subject, and the stimuli were unfamiliar to the subject at the time of presentation. The position of each stimulus set was varied randomly to control for possible position preferences within the apparatus. The total time that the subject's head was in each of the three proximity zones was recorded.

All tests were recorded with a Canon Vixia HFM31 HD camera and a Sennheiser ME66 microphone. Digital videos were coded using ELAN annotation software (http://tla.mpi.nl/tools/tla-tools/elan/) by trained assistants who were blind to the subject's treatment. In addition, all researchers were blinded to treatment throughout the experiment, until after data collection and coding was complete.

Statistical Analyses

To test the effect of the treatment on the behavioral response of chicks in the social isolation tests the day after fledging (perch hops, head saccades, and vocalizations), we used a linear mixed

model (LMM). In this model, Sex,Treatment, and Test (isolation versus with male parent) were specified as fixed factors. Random factors were individual ID (57 levels), nested within Family ID (17 levels). The interaction effect considered was Treatment X Test.

To test the effect of treatment on behavior in the four-way tests, which consisted of the number of zone changes, time spent in any proximity zone, and time spent in each proximity zone (parents, opposite sex conspecifics, or same sex conspecifics), we again used a LMM. For the model investigating the effect of treatment on the number of zone changes and time in any proximity zone, Sex, Treatment and Test Day (9 weekly tests, Day 30 to Day 86) were specified as fixed factors. Random factors were individual ID (57 levels), nested within Family ID (17 levels). The interaction effects considered were Treatment X Test Day and Sex X Treatment X Test. In the models testing the effect of treatment on time in individual proximity zones, we additionally included the time in any proximity zone as a fixed factor. We performed LMM within-Treatment to test the significance of changes across Test Day. In addition, we discuss any sex differences observed, where relevant.

All statistical analyses were performed with R software (R Development Core Team 2007). We used the *lmer* function of the lme4 package (Bates et al., 2014) which allowed us to define multiple distinct random factors. To perform model comparisons for the LMM models, we used likelihood ratio tests to compare the full model to a reduced null model with only the factor of interest removed using the *anova* function to perform a chi-square test. To test the significance of each fixed effect within a model, we used the Kenward-Roger approximation to get approximate degrees of freedom and the t-distribution to get p-values (Kenward-Roger in the pbkrtest package) and these results are presented in tables (Højsgaard, 2014). In addition, we performed post hoc tests on the interaction terms using the *testInteractions* function in phia package (Rosario-Martinez

et al., 2015).

Results:

Social Isolation Tests

A total of fifty-six fledglings were tested in the SI test. Tests were not performed for two subjects and the video files were corrupted for another two subjects. Consistent with Zann's observations, recently fledged zebra finches were relatively inactive in isolation (1996). The average number of perch hops was only 1.9 ± 3.2 perch hops per minute. However, MC birds showed the highest number of perch hops in isolation (3.3 ± 4.8 perch hops per minute) and decreased their perch hop rate when reunited with the male parent ($X^2(1) = 9.96$, $p = 0.0016$) (Fig 2a). There was a statistically significant interaction between Treatment and Test, controlling for sex and for ID nested within Family ID as random factors in a linear mixed model (LMM: $X^2(2) = 9.81$, $p = 0.0074$) (Table 1a). Post hoc tests suggest that AVT and MC birds differed from each other in their slope and there was a non-significant difference between MC and Control (AVT-MC: $X^2(1) = 9.58$, $p = 0.0059$, MC-Control: $X^2(1) = 4.99$, $p = 0.051$).

Saccadic head movements are thought to be a good proxy of visual scanning and vigilance in birds because movements of the head are necessary for birds to search for objects of interest in their environment (Fernández-Juricic, 2012). AVT birds showed the lowest rate of saccadic head movements in isolation, but showed a significant increase in the head saccade rate when the male parent was present ($X^2(1) = 6.28$, $p = 0.012$) (Fig 2b). However, neither MC nor Control birds showed a statistically significant change between isolation and the male parent test. In the overall model, there was also a statistically significant interaction between Treatment and Test (LMM: $X^2(2) = 7.00$, $p = 0.030$) (Table 1b). There was also a statistically significant effect of sex, with

males showing a slightly higher rate of saccadic head movements (Table 1b). Post hoc tests suggest that AVT and MC birds have statistically different slopes ($X^2(1) = 7.41$, $p = 0.019$).

Both AVT and Control birds vocalized at a higher rate in the presence of their male parent compared to isolation, whereas MC birds showed no change in their vocalization rate (AVT: $X^2(1) = 12.02$, $p = 0.00053$; MC: $X^2(1) = 0.83$, $p = 0.363$; Control: $X^2(1) = 12.81$, $p = 0.00035$) (Fig 2c). There was a statistically significant interaction between treatment and day (LMM: $X^2(2) = 6.99$, $p = 0.030$), with MC and Control birds differing in their slopes ($X^2(1) = 7.14$, $p = 0.023$) (Table 1c).

Four-Way Tests

All three treatment groups showed an increase in the number of zone changes (activity level) but at a decreasing rate over the course of the nine four-way tests such that the data are best modeled as a quadratic function (Fig. 3a). However, there was no difference between the treatment groups, when controlling for proportion of time in any preference zone, Sex, and ID nested within Family ID as a random effect. Similarly, there was a quadratic increase in the proportion of time spent in any preference zone across the nine four-way tests (Fig 3b). The best model for the proportion time spent in any preference zone included Treatment as a predictor, though the individual effect was not significant and there were no significant interactions between Treatment and Day (i.e. no difference in slope) (LMM: $X^2(2) = 6.83$, $p = 0.033$). Because of the change in proportion of time spent in any preference zone across days, we controlled for this total time in all future analyses. There were no sex differences in either total affiliation time or the number of zone changes.

Control subjects were the only group that decreased significantly in the time spent in the proximity zone nearest the parents across days ($X^2(2) = 6.79$, $p = 0.034$) (Fig 4). However, there was not a significant interaction between Treatment*Day, when controlling for the proportion of

time spent in any proximity zone, Sex, and ID nested within Family ID as a random effect (LMM: $X^2(4) = 3.66$, $p = 0.45$). There were also no observed sex differences in time spent in the parent proximity zone.

Control and AVT birds of both sexes showed a significant increase in time spent in the proximity zone nearest opposite sex conspecifics across test days, whereas MC birds showed no change in the time spent with opposite sex birds (AVT: X2(2) = 8.35, p = 0.015; MC: X2(2) = 0.78, $p = 0.678$; Control: $X^2(2) = 9.75$, $p = 0.0076$) (Fig 5a and 5b). However, there was a significant sex difference observed, with males and females showing a different pattern of increase in the time in the opposite sex proximity zone across development. In males, both AVT and Control males showed an increase in time spent in the female proximity zone which peaked on Day 51 before subsequently decreasing, whereas MC males showed no increase in time spent in the female proximity zone across development (LMM: $X^2(4) = 11.05$, $p = 0.026$; Fig. 5a; Table 3a). In contrast, females in all treatment groups gradually increased in the time spent in the male proximity zone, which was highly associated with the increase in total time spent in any proximity zone, but this did not differ across treatment groups (LMM: $X^2(2) = 6.15$, $p = 0.046$; Fig. 5b; Table 3b).

The best model included a Sex*Treatment*Day interaction term, though this three-way interaction was not significant in the full model (Table 2b). However, there was a significant interaction between Treatment*Day in the time spent in theopposite sex proximity zone, again controlling for total Affiliation time, Sex, and ID nested within Family ID as a random effect (LMM: $X^2(8) = 22.48$, $p = 0.0041$). Post hoc tests reveal that both AVT and Control birds were statistically different from MC birds (AVT-MC: $X^2(1) = 5.65$, $p = 0.043$, MC-Control: $X^2(1) = 5.98$, $p = 0.43$).

Neither Control nor AVT birds showed a change in the time spent in the same sex proximity

zone across the test days (Fig. 6a and 6b). However, there was a highly significant quadratic effect of Day in the MC group ($F_{1,171}$ = 13.06, p = 0.00040), with a peak in time with same sex conspecifics on Day 51 by females in the MC group only (LMM: $X^2(2)$ = 12.59, p = 0.0018; Fig 6b, Table 3d). In the full model, there was highly significant interaction between Treatment*Day (LMM: X^2 (8) = 32.21, p = 8.55 x 10^{-5}), with the best model including a Sex*Treatment*Day interaction term, controlling for total time spent in any proximity zone, Sex, and ID nested within Family ID as a random effect (Table 2c). Post hoc tests indicated that AVT and MC birds were statistically different from each other ($X^2(1)$ = 6.89, p = 0.026).

Discussion:

To our knowledge, these results provide the first evidence that nonapeptides are involved in social development in a songbird species. Compared to control subjects, birds that had V1a (and potentially VT3/OT-like) receptors antagonized early in life showed a significantly altered behavioral pattern throughout development. As fledglings, MC subjects were twice as active in isolation and in fact decreased their activity levels when in the presence of the male parent. In fact, MC subjects were generally unresponsive to the male parent, with no change in the number of vocalizations emitted in the presence of the male parent compared to isolation and no change in their visual scanning rate. In the four-way affiliative preference tests, MC birds were slightly more affiliative over all, but they demonstrated no preference for their parents versus unfamiliar conspecifics at any point in development and neither male nor female MC birds showed an increase in interest in opposite sex individuals as they matured.

AVT birds, on the other hand, seemed to show the most specific affiliative behaviors throughout development, though they were more similar to controls than MC birds were. AVT

subjects were less active overall in isolation than both control and MC birds, and showed an increase in both visual scanning rate and vocalizations when in the presence of the male parent. Consistent with our predictions, they also showed elevated preferences for parents throughout development, affiliating with parents more than expected by random chance in 7 of 9 periods (Control and MC subjects showed this pattern in only 4 of 9 periods). AVT males also showed a strong increase in affiliative preference for opposite sex birds early in development similar to controls.

There was one unexpected sex difference to emerge from this study. Both AVT and Control males showed an increase in affiliative interest in females (opposite sex) which peaked on Day 51, but this increase was absent in the MC males. Surprisingly, MC females showed a pattern of increased interest in females (same sex) that mimic the pattern observed in males. This raises the intriguing possibility that MC females were somehow masculinized in their affiliative interest, hinting at opposing effects of MC in males and females, though MC females did not appear to show any other masculinized behaviors. Nevertheless, these results suggest that the nonapeptide receptors, and specifically V1aR, may be involved in mediating affiliative interest in female stimuli, which is consistent with the role of AVT in appetitive, courtship, and sexual behaviors across taxa (Boyd, 2013; Castagna et al., 1998; Godwin and Thompson, 2013; Goodson et al., 2004; Smock et al., 1998).

In this study, intracranial injections of AVT or a V1aR/OTR antagonist did not affect growth or survival in chicks prior to fledging. We did not directly assess feeding or drinking behavior, but there were no obvious differences between groups in activity levels during the four-way tests. The lack of such an impact may be because the injections appear to influence primarily non-hypothalamic AVT-sensitive cell groups, minimizing detrimental consequences related to

water balance and thermoregulation.

However, one possible explanation for the observed effects of the experimental manipulations on social behavior could be that the injections affect the regulation of the hypothalamic-pituitary-adrenal (HPA) axis. AVP/AVT serves as a releasing factor, along with corticotropin-releasing factor (CRF), for the production of adrenocorticotropic hormone (ACTH) in the anterior pituitary (Buckingham, 2009). Perch hops and other measures of activity level have been found to be correlated with HPA activity and anxiety in zebra finches (Remage-Healey et al., 2008; Schweitzer et al., 2014; Woodgate et al., 2010). Thus, it is possible that the higher activity level in MC birds during the social isolation tests were indicative of a change in the HPA-related functions of AVT throughout development. However, it is important to note that there were no differences between treatment groups in the number of zone changes during the four-way tests.

Instead, these results suggest that nonapeptides in the brain play an important role during sensitive periods in development and are involved in altering the neural pathways necessary for species-typical social behavior. It has long been recognized that both humans and animals of many different taxa show social attachments in which separation leads to increased feelings of stress or anxiety (Carter, 1998; Panksepp et al., 1997). The social isolation test used in the present study is in many ways analogous to the "Strange Situation" used to assess social attachment in human infants (Ainsworth and Bell, 1970). In this test, attachment to the caregiver is assessed by measuring the behavioral response of the infant to separation from the caregiver, the presence of a stranger, and then reunion with the caregiver. Secure attachment is characterized by exploration and secure-base behavior when the caregiver is present, a marked increase in anxiety when separated from the caregiver, and then a reduction in distress upon their return. Insecurely attached infants, particularly the anxious-avoidant subtype, seem indifferent to the presence of the caregiver

and do not exhibit typical stranger anxiety.

Although we did not directly test the response of the juveniles to an unfamiliar conspecific in the social isolation tests, the subjects could hear and interact vocally with the unfamiliar birds located in the room. Both AVT and control subjects showed a low vocalization rate and were less active in isolation compared to when the male parent was present, a pattern typical of secure attachment, as well as evidence of the ability to distinguish between the vocalizations of the parents and unfamiliar conspecifics. Based on the life history of the zebra finch, we would also predict this pattern of minimal activity in the absence of parents, followed by an increase in vocalizations and activity level when the parents return (Zann, 1996). In contrast to this expected pattern, MC subjects vocalized more during this isolation from the family than AVT birds and were more active than both control and AVT juveniles.

Thus, there are several possible interpretations of MC subjects' behavior during isolation. One possibility is that they are simply more distressed by separation from their parents and family and are actively searching for their parents and family. However, their increased activity level in absence of their parents may also be indicative of a weaker, less specific, or even insecure attachment to their parents. It is possible, for example, that they are more active because they have not developed the ability to distinguish between the calls of their parents and the unfamiliar adults that are in the room (i.e. the strangers), and thus locomote and vocalize more in response to unfamiliar adult distance calls. This is consistent with observations that the specificity of the fledgling responses to adult distance calls seems to develop over time (Mulard et al., 2010; Zann, 1996). Alternatively, MC subjects may simply not be distressed by separation from their parents, and are exhibiting more exploratory behaviors. Our data do not allow us to distinguish between these possibilities because neither vocalizations nor locomotor activity have been definitely linked

to stress or anxiety in juvenile zebra finches. However, the data from the four-way affiliative preference tests suggests that the most parsimonious explanation is that MC has somehow affected the specificity of the attachment to the parents, though we cannot rule out that this effect is not mediated by generalized effects on HPA axis regulation. Nevertheless, our results are consistent with the idea that zebra finch juveniles form specific affiliative bonds with the parents and also provide the first evidence that this attachment relationship may be mediated by the nonapeptide system in birds.

There are several possible mechanisms by which these alterations of the nonapeptides early in development may have changed the processing of social information. One possibility is that AVT and MC have permanently altered social recognition abilities in the zebra finches. The nonapeptide system is known to be very important in social recognition in rodents. For example, oxytocin knockout mice fail to recognize familiar conspecifics and infusion of oxytocin into the medial amygdala restores social recognition (Ferguson et al., 2001, 2000). Neurons in the meAMY also show selectivity to stimulus odors from conspecifics of different sexes, as well as a striking sexual dimorphism in processing of olfactory information (Bergan et al., 2014). Mice with a null mutation for V1aR display a profound impairment in social recognition, which can be restored by re-expression of V1aR in the lateral septum (Bielsky et al., 2004). Furthermore, viral vector-mediated gene transfer of the prairie vole V1aR into the lateral septum of the rat led to a dramatic improvement in social discrimination (Landgraf et al., 2003).

Zebra finch fledglings typically respond exclusively to the distance calls of their parents within a short time after fledging, suggesting that the specificity of this vocal response develops with experience (Mulard et al., 2010). The present finding that MC birds emit significantly more long tonal calls in the absence of their parent and show no preference for parents even at Day 30 suggest

that MC birds may be either impaired or delayed in their recognition of their parents. Indeed, it is possible that MC injected birds suffer from an impairment in social recognition more broadly and have a more difficult time differentiating between the identities of different individuals that they encounter because of changes to how nonapeptides modulate these critical brain regions.

Another potential explanation is that MC subjects do not have any impairment in recognition per se, but have an impairment in assigning value or importance to different individuals. This interpretation would be consistent with the observation that the AVT neurons in the extended medial amygdala are sensitive to social valence in mammalian species (Newman, 1999; Sheehan et al., 2001). In zebra finches, AVT neurons in the BSTm have been found to be sensitive to social valence (Goodson et al., 2009, 2004; Goodson and Wang, 2006). There is also evidence that neurons in the extended medial amygdala are involved in the formation and maintenance of pair bonds in both zebra finches and prairie voles (Curtis and Wang, 2003; Svec et al., 2009). The extended medial amygdala and the lateral septum are integral parts of both the social behavior network and the mesolimbic reward network, so it is also possible that the present results are a function of alterations to the nonapeptide-sensitive neurons in these regions which are thought to be critical for assigning a reward value or valence to conspecifics (O'Connell and Hofmann, 2011).

A third explanation is that the early manipulations of the AVT system alter attentiveness to or the salience of social stimuli only at the time of injection, leading to downstream differences in socially-relevant behaviors later in life. In this view, AVT does not directly alter the production of or sensitivity to nonapeptides within the brain. Instead, by acting in regions that process social information, AVT may act to increase the salience of these social stimuli at a sensitive point during learning, such that the developing bird learns to more strongly or easily form an association between features of their parents and reward. Thus, changes to the AVT system, coupled with

salient social experiences occurring at the time of the injections, could lead to changes in social behaviors via domain-general learning mechanisms. Of course, these possible explanations are not mutually exclusive, but further research is needed to determine the most likely mechanisms.

Unfortunately, a major challenge for interpreting the present results is the lack of detailed comparative information about where and when nonapeptides and their receptors are expressed in the developing brain. Detailed experiments have outlined the ontogeny of the nonapeptide systems in rats, which demonstrate that the production of AVP by the hypothalamus begins during fetal development. AVP can be labeled in the SON and PVN by fetal day 14 (gestation is 21 days in rats), reaching adult levels by postnatal day 30 (Buijs et al., 1980; Szot and Dorsa, 1993). The production of AVP mRNA in the meAMY and BSTm begins later in development (between postnatal day 3 and day 14) and is highly sexually dimorphic, with production of AVP starting later in females and taking longer to reach adult levels (Szot and Dorsa, 1993). Additionally, binding sites were found in the developing rodent brain in both the amygdala and septum between postnatal day 0 and 8, as well as several brain regions where AVP receptors are not expressed in adulthood, including the hippocampus, dentate gyrus, and caudate nucleus (mice: Hammock et al., 2013; rat: Petracca et al., 1986; multiple vole species: Wang et al., 1997). AVT is produced in the brain at least as early as 4 weeks of age in male canaries, though earlier ages were not assessed (Voorhuis et al., 1991). In zebrafish, both AVT and the two teleost V1a receptor subtypes are expressed together very early in development, implicating AVT in the development of the nervous system or control of early behavior across vertebrates generally (Eaton et al., 2008; Iwasaki et al., 2013).

Additionally, experimental evidence from rodents does provide evidence consistent with the hypothesis that nonapeptides may modulate many brain regions early in development, particularly

those relevant for social behavior. In rats, the beginning of production of AVP/OT from the extrahypothalamic sources and central nonapeptide receptor expression is coincident with important milestones in early social attachment and learning in rat pups (Blass, 1987; Buijs et al., 1980; Hammock et al., 2013; Petracca et al., 1986; Szot and Dorsa, 1993). A limited number of experimental manipulations of nonapeptides during development in rodents provide further evidence for the organizational hypothesis. Vasopressin-deficient Brattleboro rat pups show hyperactivity, reduced huddling and reduced proximity to other pups in the nest compared to wild-type rats (Schank, 2009). Wild-type rat pups treated with a nine-day exposure to AVP showed increased emotionality, activity levels and grooming in an open field test as juveniles, as well as smaller overall brain size (Boer et al., 1994). Acute central administration of AVP in wild-type neonatal rat pups was found to decrease the number of ultrasonic vocalizations and reduced locomotor activity in a maternal isolation test (Winslow and Insel, 1993). In juvenile male rats, both targeted infusion of AVP into the LS and intracerebroventricular infusion increased preference for investigating novel individuals, whereas a V1aR antagonist increased the preference for investigating familiar individuals (Veenema et al., 2012). In addition, V1aR blockade in the LS increased social play behavior in males and decreased it in females, but only when it tested in a familiar environment (Bredewold et al., 2014; Veenema et al., 2013). In addition, neonatal manipulation of AVT or OT in the socially-monogamous prairie vole, leads to significant changes in nonapeptide binding in several brain regions in adults and alterations to social behaviors (Bales et al., 2007; Bales and Carter, 2003; Stribley and Carter, 1999; Yamamoto et al., 2004).

Thus, our results are consistent with the idea of a similar organizational role for nonapeptides in social development in zebra finches. However, it is important to note that the time between the end of the injections and behavioral testing in the present experiment was as little as 8 days,

although it was over 12 weeks by the end of the four-way tests. While the influences of the early injections on affiliative preferences after sexual maturity are much more likely to be organizational effects, it is possible that the results observed in the SI tests represent direct effects on the activation of the nonapeptide system in juveniles. Furthermore, we did not test the effect of nonapeptide manipulations outside of the week window shortly after hatching, so we cannot definitively conclude that effects of this magnitude would only occur at this point in development. However, intracerebroventricular manipulations of the nonapeptide system using multiple day manipulations of similar dosages did not yield significant effects on affiliative behavior in adults (Goodson et al., 2004). Additionally, we have not tested for whether the effects are either permanent or reversible, so further research is needed to confirm that these effects can indeed be considered organizational.

A further challenge, which remains a challenge for the study of nonapeptides generally, is the issue of receptor specificity. Because nonapeptide receptor subtypes have high degrees of structural homology with each other and each subtype is highly variable across species, we can infer that both AVT and MC will bind to multiple receptor subtypes in the zebra finch brain with unknown affinities (Leung et al., 2011, 2009). Although MC most potently targets V1aR, it also serves as a mild antagonist for OT-like receptors as well (Kruszynski et al., 1980; Leung et al., 2009; Manning et al., 2012). To complicate matters further, several authors have speculated that AVT (not MT) may in fact be the endogenous ligand of the avian VT3 (OT-like) receptor both inside and outside the brain because AVT may actually have a higher affinity for VT3 receptors than MT (Baeyens and Cornett, 2006; Gubrij et al., 2005). Thus, AVT may be the more relevant endogenous ligand for a number of social functions via its action at multiple receptor subtypes, despite evidence that alterations of MT affect behavior (Kelly and Goodson, 2014b; Lowrey and Tomaszycki, 2014). Thus, further research is needed to determine whether the observed effects are

a result of the actions of AVT and MC at V1a receptors, VT3 (OT-like) receptors, or, more likely, both.

Conclusion:

These results provide evidence that the nonapeptide system, particularly AVT and V1aR, play an important role in organizing the neural pathways necessary for species-typical affiliative behaviors and attachment in zebra finches. The nonapeptides have been identified as important mediators of individual and species differences in social behaviors more broadly (Goodson, 2005). These results provide support for the idea that changes to the nonapeptide system during development may in fact be an important mechanism underlying the evolution of species-differences in social phenotypes (Syal and Finlay, 2011). Much additional research is needed, but these results suggest that explorations of the evolution and development of the nonapeptide systems will prove fruitful.

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Figures

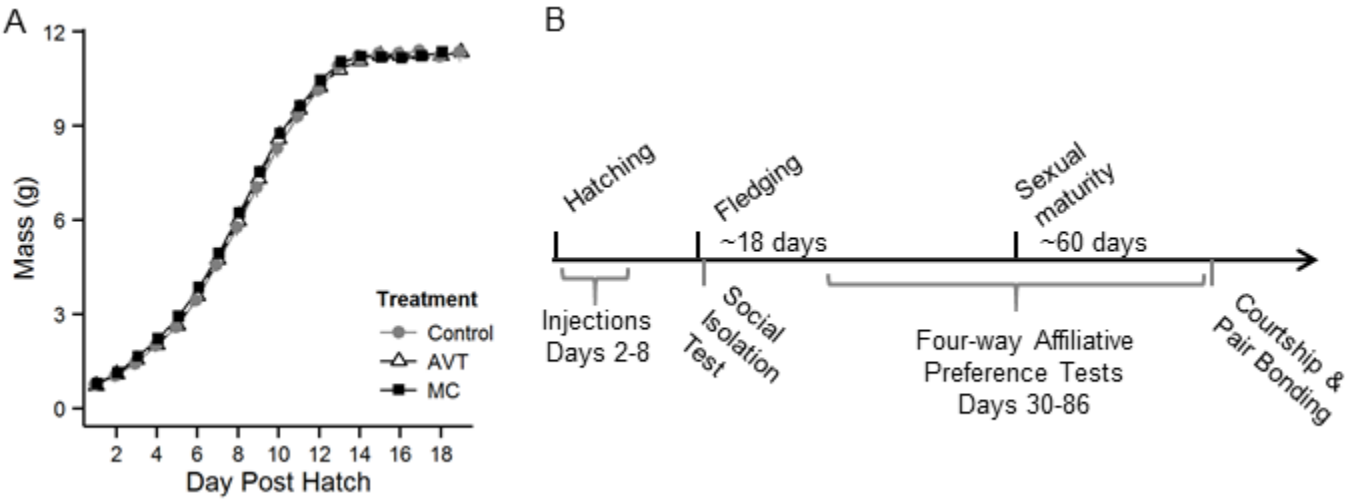

Figure 4: Growth curve and Experimental Timeline

Panel A) depicts the mean ± SE chick mass (g) for each day post-hatching until fledging. Control subjects are depicted with circles and a solid gray line, AVT with triangles and dashed black line, and MC as squares and solid black line. The growth curves are highly overlapping across treatments and there are no statistically significant differences between groups. Panel B) shows the experimental timeline with developmental events (hatching, fledging, and timing of reproductive maturity) above the line and experimental events (injections, social isolation tests, and four-way affiliative preference tests) below the line.

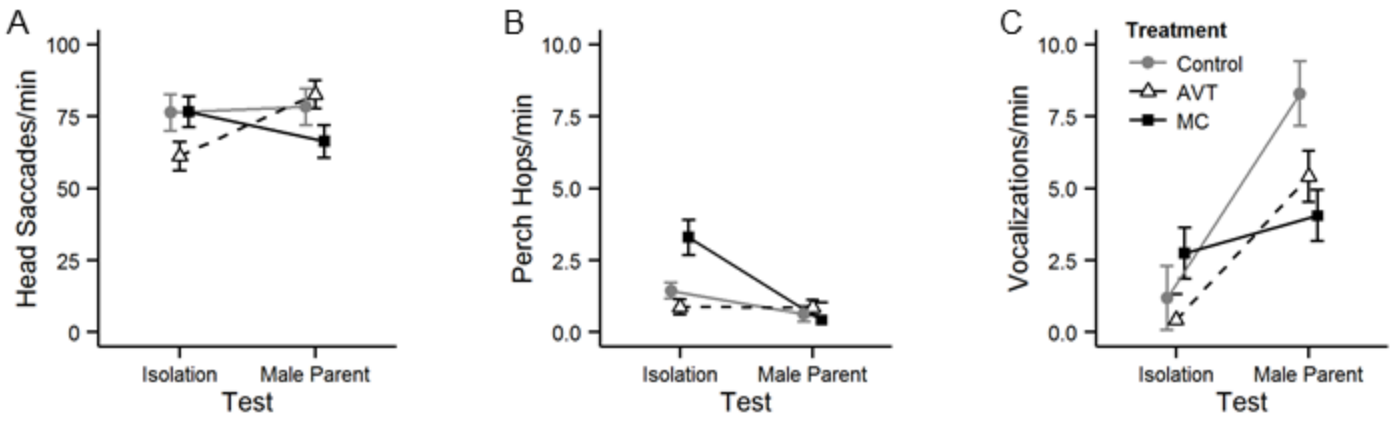

Figure 5: Saccadic head movements, perch hops and vocalizations in social isolation tests

Mean ± SE of the number of A) saccadic head movements, B) perch hops, and C) vocalizations per minute in the social isolation test in isolation compared to when the male parent was present. Control subjects are depicted with circles and a solid gray line, AVT with triangles and dashed black line, and MC as squares and solid black line.

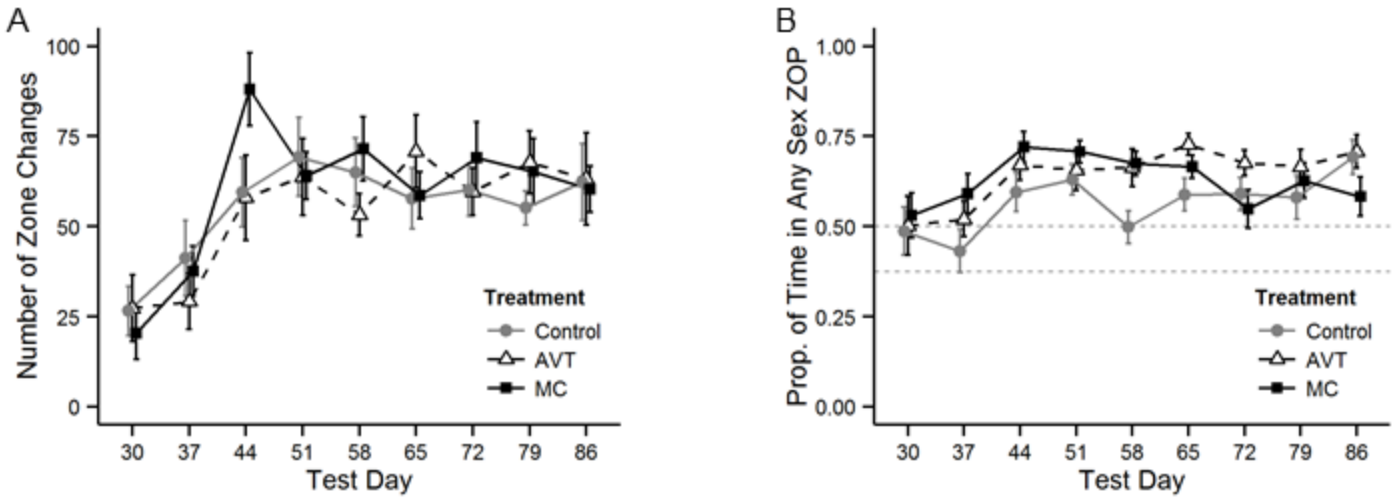

Figure 6: Zone changes and time spent in any zone of proximity (ZOP)

Mean ± SE of A) the number of zone changes across testing days during the four-way affiliative preferences tests, and B) the proportion of total test time spent in any of the three zones of proximity (ZOP, as opposed to the neutral zone). In panel B, the dashed gray horizontal lines depict the boundaries delineating what can be considered random activity in the apparatus (3/8 if determined by perch use or 1/2 if by total area use). Thus, means outside the boundary of these lines suggest a preference that differs from random chance. Control subjects are depicted with circles and a solid gray line, AVT with triangles and dashed black line, and MC as squares and solid black line.

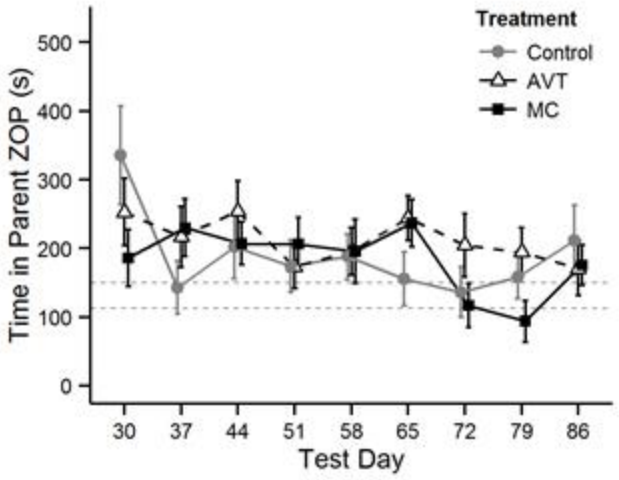

Figure 7: Affiliative preference for parents in male and female subjects

Mean ± SE of the time in seconds spent in the zone of proximity (ZOP) with the male and female parent across testing days during the four-way affiliative preferences tests with both sexes of subjects combined. Each four-way test was 900 seconds long. In both panels, the dashed gray horizontal lines depict the boundaries delineating what can be considered random activity in the apparatus (112.5s if determined by perch use or 150s if by total area use). Thus, means outside the boundary of these lines suggest a preference that differs from random chance. Control subjects are depicted with circles and a solid gray line, AVT with triangles and dashed black line, and MC as squares and solid black line.

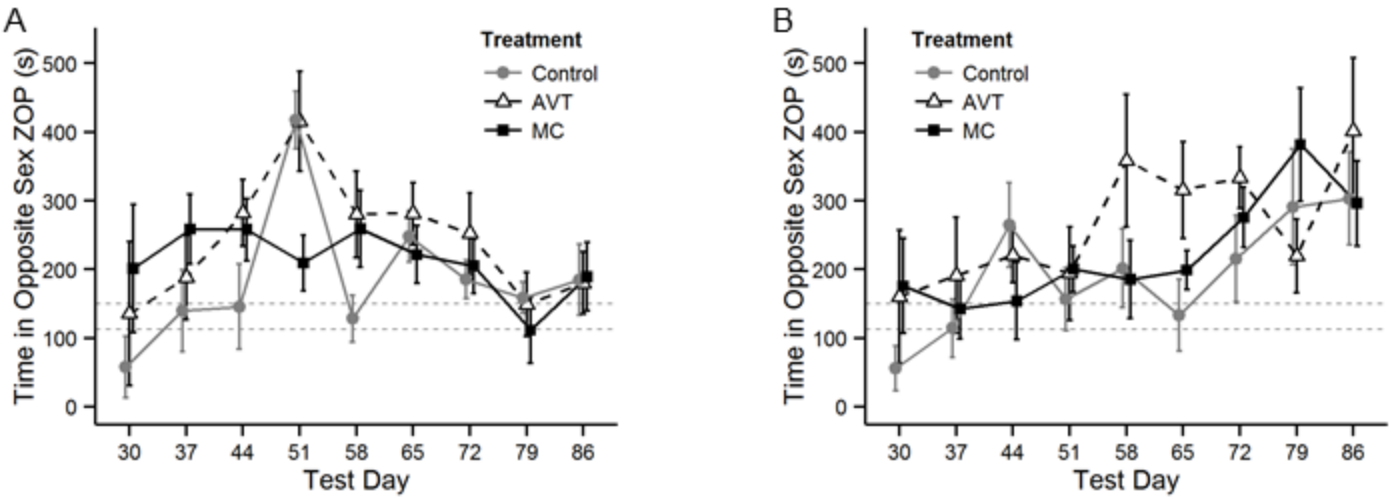

Figure 8: Affiliative preference for opposite sex conspecifics in male and female subjects

Mean ± SE of the time in seconds spent in the zone of proximity (ZOP) with two opposite sex conspecifics across testing days during the four-way affiliative preferences tests in A) male subjects and B) female subjects. Each four-way test was 900 seconds long. In both panels, the dashed gray horizontal lines depict the boundaries delineating what can be considered random activity in the apparatus (112.5s if determined by perch use or 150s if by total area use). Thus, means outside the boundary of these lines suggest a preference that differs from random chance. Control subjects are depicted with circles and a solid gray line, AVT with triangles and dashed black line, and MC as squares and solid black line.

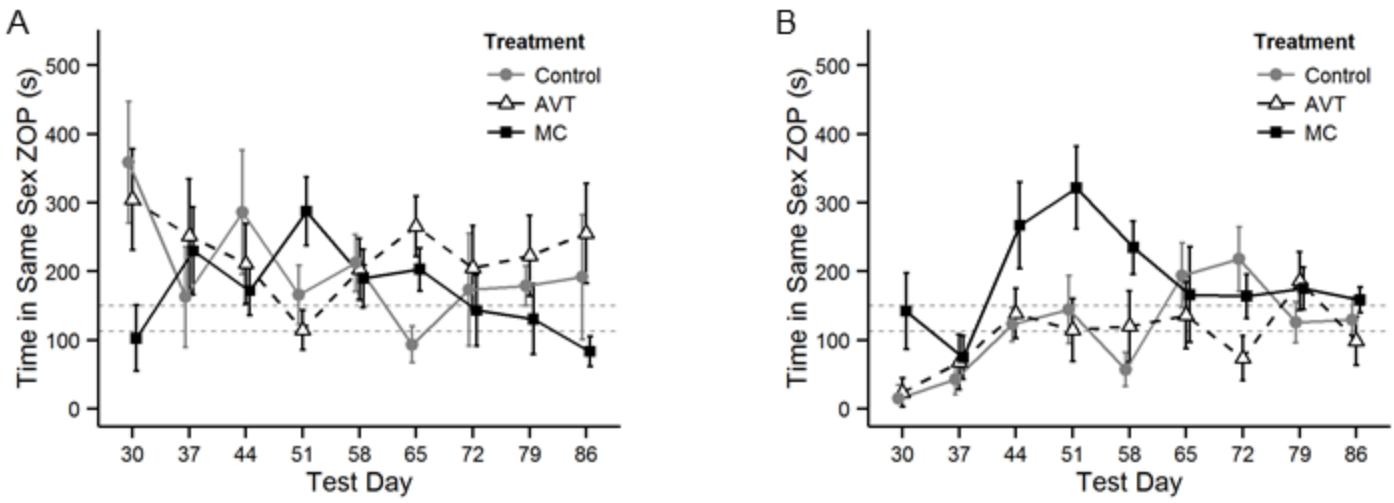

Figure 9: Affiliative preference for same sex conspecifics in male and female subjects

Mean ± SE of the time in seconds spent in the zone of proximity (ZOP) with two same sex conspecifics across testing days during the four-way affiliative preferences tests in A) male subjects and B) female subjects. Each four-way test was 900 seconds long. In both panels, the dashed gray horizontal lines depict the boundaries delineating what can be considered random activity in the apparatus (112.5s if determined by perch use or 150s if by total area use). Thus, means outside the boundary of these lines suggest a preference that differs from random chance. Control subjects are depicted with circles and a solid gray line, AVT with triangles and dashed black line, and MC as squares and solid black line.

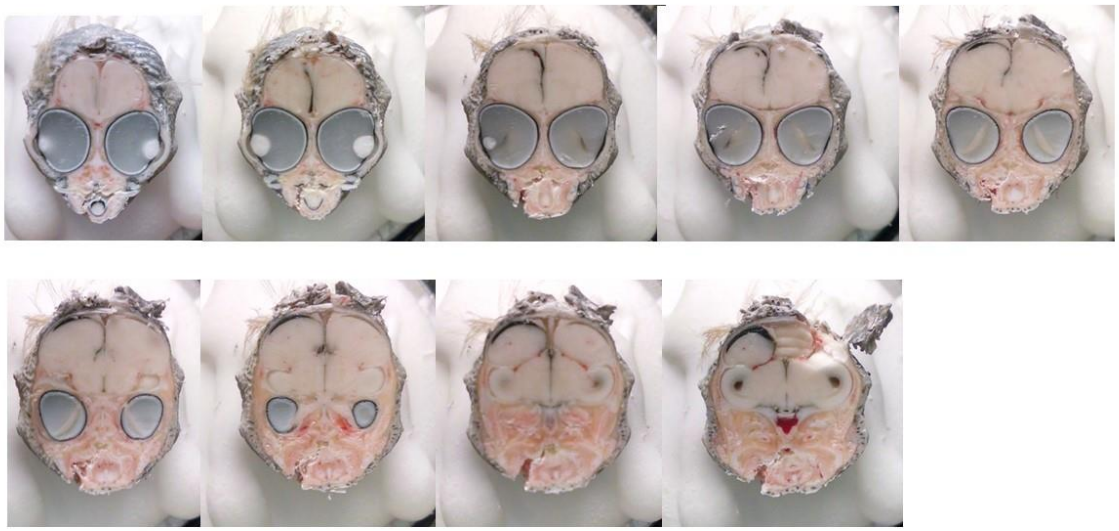

Figure 10: India ink staining of intracranial injections

India ink staining in the head (including skull) of a Day 8 chick four hours following intracranial injection. Staining is present primarily in the right lateral ventricle, with more limited staining in the third ventricle. The top left photo is the most rostral with subsequent images moving further caudal.

Tables

	Perch Hops			
Predictors	Estimate	SE	*t*	*p*
Intercept	1.181	0.556	2.125	**0.037**
Sex (Male)	0.479	0.408	1.174	0.244
Treatment (AVT)	-0.593	0.679	-0.874	0.385
Treatment (MC)	1.766	0.690	2.560	**0.012**
Test (Male Parent)	-0.791	0.678	-1.166	0.247
Treatment (AVT)* Test(Male Parent)	0.765	0.947	0.808	0.422
Treatment (MC)*Test (Male Parent)	-2.089	0.935	-2.234	**0.028**

	Head Saccades			
	Estimate	SE	*t*	*P*
Intercept	67.328	8.796	7.654	**7.52E-12**
Sex (Male)	15.117	6.801	2.223	**0.028**
Treatment (AVT)	-15.412	10.216	-1.509	0.134
Treatment (MC)	1.591	10.673	0.149	0.882
Test (Male Parent)	2.063	9.308	0.222	0.825
Treatment (AVT)* Test(Male Parent)	19.391	12.634	1.535	0.128
Treatment (MC)*Test (Male Parent)	-13.783	12.736	-1.082	0.281

	Vocalizations			
	Estimate	SE	*t*	*p*
Intercept	0.824	1.253	0.658	0.513
Sex (Male)	0.785	0.946	0.830	0.410
Treatment (AVT)	-0.797	1.613	-0.494	0.623
Treatment (MC)	1.533	1.593	0.962	0.340
Test (Male Parent)	7.101	1.595	4.451	**0.000**
Treatment (AVT)* Test(Male Parent)	-2.120	2.196	-0.965	0.338
Treatment (MC)*Test (Male Parent)	-5.800	2.170	-2.673	**0.010**

Table 2: Social isolation test linear mixed model (LMM) results

Summary of the linear mixed models with the number of saccadic head movements, perch hops, and vocalizations performed by the subject as the dependent variables. The fixed effects of Sex, Treatment, Test (Isolation versus Male Parent) as well as the interactions are shown. Individual ID nested within Family ID was included as a random effect. To test the significance of each parameter within the model, we used the Kenward-Roger approximation to get approximate degrees of freedom and the *t*-distribution (SE = standard error, bold numbers indicate significance, * refers to an interaction term).

	Parents				*Opposite Sex*				*Same Sex*			
Predictors	Estimate	SE	*t*	*p*	Estimate	SE	*t*	*p*	Estimate	SE	*t*	*p*
Intercept	111.879	38.260	2.924	**0.004**	-71.131	70.852	-1.004	0.316	-121.231	65.558	-1.849	0.065
Total Affiliation Time	311.852	30.691	10.161	**0.00E+00**	398.042	32.394	12.288	**0.00E+00**	227.494	26.593	8.555	**6.66E-16**
Sex	9.587	18.513	0.518	0.605	-168.186	105.444	-1.595	**0.112**	355.901	97.257	3.659	**2.99E-04**
Day	-39.597	12.846	-3.082	**0.002**	7.599	30.548	0.249	0.804	51.692	27.317	1.892	0.059
Day^2	2.659	1.231	2.159	**0.032**	0.339	2.928	0.116	0.908	-4.460	2.613	-1.707	0.089
Treatment(AVT)					56.859	97.918	0.581	0.562	23.497	90.531	0.260	0.795
Treatment(MC)					7.552	96.226	0.078	0.937	53.197	88.837	0.599	0.550
Sex*Treatment(AVT)					26.602	142.089	0.187	0.852	-36.890	131.116	-0.281	0.779
Sex*Treatment(MC)					258.159	140.782	1.834	0.068	-282.676	129.748	-2.179	**0.030**
Sex*Day					87.482	47.198	1.853	0.065	-124.060	42.030	-2.952	**3.41E-03**
Sex*Day^2					-9.341	4.589	-2.036	**0.043**	10.399	4.086	2.545	**0.011**
Treatment(AVT)*Day					-9.810	43.576	-0.225	0.822	-29.398	38.894	-0.756	0.450
Treatment(MC)*Day					-34.375	43.436	-0.791	0.429	6.254	38.685	0.162	0.872
Treatment(AVT)*Day^2					0.836	4.209	0.199	0.843	2.665	3.748	0.711	0.478
Treatment(MC)*Day^2					4.515	4.238	1.065	0.287	-1.377	3.771	-0.365	0.715
Sex*Treatment(AVT)*Day					7.855	63.615	0.123	0.902	32.555	56.668	0.574	0.566
Sex*Treatment(MC)*Day					-85.854	63.521	-1.352	0.177	101.847	56.530	1.802	0.073
Sex*Treatment(AVT)*Day^2					-1.966	6.181	-0.318	0.751	-2.132	5.499	-0.388	0.698
Sex*Treatment(MC)*Day^2					6.371	6.192	1.029	0.304	-9.191	5.505	-1.670	0.096

Table 3: Linear mixed model (LMM) results for four-way affiliative preference tests

Summary of the linear mixed models with the time spent in the parent zone of proximity (ZOP), opposite sex ZOP, or same sex ZOP in the four-way affiliative preference tests as the dependent variables. The fixed effects are Total Affiliation Time (Time in any ZOP), Sex, Day, Day^2, Treatment, as well as the interactions. Individual ID nested within Family ID was included as a random effect. The LMM models were selected based on model comparisons using likelihood ratio tests. To test the significance of each parameter within the models, we used the Kenward-Roger approximation to get approximate degrees of freedom and the *t*-distribution (SE = standard error, bold numbers indicate significance, * refers to an interaction term).

Opposite Sex	Males				Females			
Predictors	Estimate	SE	*t*	*p*	Estimate	SE	*t*	*p*
Intercept	-227.842	85.659	-2.660	**8.57E-03**	-59.679	49.796	-1.198	0.232
Total Affiliation Time	369.021	44.700	8.255	**4.11E-14**	436.845	45.877	9.522	**0.00E+00**
Day	95.677	35.724	2.678	**8.13E-03**	-8.563	18.181	-0.471	0.638
Day^2	-8.984	3.515	-2.556	**0.011**	2.131	1.709	1.247	0.214
Treatment(AVT)	75.109	106.968	0.702	0.484	20.237	36.358	0.557	0.578
Treatment(MC)	288.610	106.503	2.710	**7.42E-03**	-40.737	36.442	-1.118	0.265
Treatment(AVT)*Day	0.438	45.984	0.010	0.992				
Treatment(MC)*Day	-120.512	46.056	-2.617	**0.010**				
Treatment(AVT)*Day^2	-1.284	4.502	-0.285	0.776				
Treatment(MC)*Day^2	10.653	4.512	2.361	**0.019**				

Same Sex	Estimate	SE	*t*	*p*	Estimate	SE	*t*	*p*
Intercept	10.632	27.149	0.392	0.696	-68.711	34.435	-1.995	0.047
Total Affiliation Time	289.513	40.075	7.224	**3.05E-11**	147.461	32.901	4.482	**1.26E-05**
Day					48.201	13.849	3.481	**6.18E-04**
Day^2					-4.305	1.339	-3.214	**1.53E-03**
Treatment(AVT)					-24.643	21.720	-1.135	0.258
Treatment(MC)					56.980	21.915	2.600	**0.010**
Treatment(AVT)*Day								
Treatment(MC)*Day								
Treatment(AVT)*Day^2								
Treatment(MC)*Day^2								

Table 4: Linear mixed model (LMM) results for four-way affiliative preference tests for opposite and same sex conspecifics within sex

Summary of the linear mixed models with the time spent in the opposite sex zone of proximity (ZOP) (top panel) or same sex ZOP (bottom panel) in the four-way affiliative preference tests as the dependent variables. The fixed effects are Total Affiliation Time (Time in any ZOP), Day, Day^2, Treatment, as well as the interactions. Individual ID nested within Family ID was included as a random effect. The LMM models were selected based on model comparisons using likelihood ratio tests. To test the significance of each parameter within the models, we used the Kenward-Roger approximation to get approximate degrees of freedom and the *t*-distribution (SE = standard error, bold numbers indicate significance, * refers to an interaction term).

CHAPTER FOUR

Organizational effects of vasotocin and V1aR antagonist on affiliative behavior, pair bonding, and the extended medial amygdala in the zebra finch

Abstract: Adult zebra finches (*T. guttata*) form socially-monogamous pair bonds characterized by proximity, vocal communication, and contact behaviors. This research tests the hypothesis that the nonapeptide arginine vasotocin (AVT, avian homologue of vasopressin) and the V1a receptor subtype (V1aR) play organizational roles in species-typical affiliative behavior and pair bonding. Zebra finch hatchlings of both sexes received intracranial injections (post-hatch days 2-8) of AVT, Manning Compound (MC, a V1aR antagonist) or a saline control. On post-hatch day 90, subjects were introduced to an unmanipulated opposite sex bird and allowed to pair for seven days. We measured courtship and pair maintenance behaviors each day for one week. On the seventh day of cohabitation with the partner, we used a mate separation and reunion paradigm to induce the expression of pair maintenance behaviors. We then used double-label fluorescence *in situ* hybridization to test whether administration of AVT or MC altered the adult distribution of neurons expressing V1aR in two structures in the extended medial amygdala [the nucleus taeniae (TnA) and the medial bed nucleus of the stria terminalis (BSTm)] and whether those same neurons were active during pair maintenance behaviors by quantifying the expression of an immediate early gene, ZENK (the avian homologue of Egr-1). There was no effect of treatment on pairing behaviors in females. However, AVT males were significantly more affiliative with their female partner following separation, perching in contact with their partner nearly eight times as much as Controls. Control males, on the other hand, sang significantly more than both AVT and MC males when reunited with their female partner. AVT males had higher V1aR

expression in the TnA than both Control and MC males and immediate early gene activity of V1aR neurons in the TnA was positively correlated with affiliation. MC males showed decreased ZENK expression in the TnA. In addition, there was a negative correlation between the activity of V1aR cells in the BSTm and singing. These results provide the first evidence that AVT and V1aR play organizational roles in both the development of pair bonding behaviors and the neural substrate underlying these behaviors in a bird.

Introduction:

In species with parental care, the formation and maintenance of selective affiliative relationships begins early in an individual's life. Yet we know very little about the development of the neural and neuroendocrine mechanisms underlying the relationships offspring have with their parents, particularly how they influence the formation and maintenance of pair bonds in adulthood. Selective affiliation, or attachment, is commonly defined as an exclusive social or emotional bond, which can be measured as maintenance of proximity, voluntary contact, or differential behaviors towards the attachment object (Ainsworth, 1989; Carter et al., 1995). Many animals that exhibit pair bonding in adulthood also have a close affiliative relationship with their parents early in life—they are attached to their parents and siblings. In these species, young seem to transition from an exclusive close relationship with the family to an adult pair relationship similarly characterized by attachment and affiliation.

Nonapeptides and pairing behaviors

Nonapeptides in the oxytocin family [mesotocin (MT) and arginine vasotocin (AVT) in birds; oxytocin (OT) and arginine vasopressin (AVP) in mammals] have been implicated as important modulators of social behaviors, including pair bonding and affiliative behaviors, across taxa (Choleris et al., 2013). The primary sources of nonapeptides derive from the AVP/OT cell groups of the supraoptic (SON) and paraventricular (PVN) nuclei of the hypothalamus, as well as from smaller extrahypothalamic accessory cell groups, including the medial amygdala (MeA), medial bed nucleus of the stria terminalis (BSTm), lateral septum (LS), olfactory bulb (OB), and suprachiasmatic nucleus (SCN) (Choleris et al., 2013; Laycock, 2009).

Nonapeptides produced by these cell groups modulate the activity of neurons by subsequent binding to receptors distributed throughout the brain, but importantly the distribution of these receptors and, thus, their functions are remarkably variable across species (Goodson, 2005; Kelly & Goodson, 2014b). Neurons that contain receptors for AVT/AVP have been shown to be important in social recognition (Ferguson et al., 2001; Ferguson et al., 2000); appetitive, courtship, and sexual behaviors (Kondo & Arai, 1995; Lehman & Winans, 1982; Lehman et al., 1980; Smock et al., 1998), as well as pair formation (Curtis & Wang, 2003; Kirkpatrick et al., 1994). The abundance of AVT/AVP elements is often sexually dimorphic (usually greater expression in males compared to females), organized by sex steroids during development, and sensitive to changes in gonadal state (De Vries & al-Shamma, 1990; De Vries & Buijs, 1983; De Vries & Panzica, 2006; Goodson & Bass, 2001; Goodson & Thompson, 2010; Kabelik et al., 2010; Kelly & Goodson, 2014; Kimura et al., 1999).

Until recently, the only vertebrate species for which substantial progress has been made in uncovering the mechanisms for pairing is the prairie vole (*Microtus ochrogaster*) (Insel & Young, 2001; McGraw & Young, 2010; Young et al., 1998). Prairie voles exhibit a number of behaviors characteristic of or associated with monogamy, including preferential mating with one partner, contact behaviors, affiliative partner preference, and biparental care of young (Carter & Getz, 1993; Dewsbury, 1987; Getz & Carter, 1996; Insel et al., 1995; McGuire & Novak, 1984; Oliveras & Novak, 1986; Williams et al., 1992; Winslow et al., 1993). AVP mediates pairing in male prairie voles (Pitkow et al., 2001; Winslow et al., 1993), while pair formation is facilitated by OT in females (Williams et al., 1994), though both AVP and OT can increase affiliative behaviors in both sexes at high enough doses (Cho et al., 1999).

However, it is birds—not mammals—that are the true exemplars of social monogamy, with up to 90% of avian species exhibiting socially monogamous pair bonds (Bennett & Owens, 2002). Zebra finches (*Taeniopygia guttata*), like prairie voles, exhibit socially monogamous pair bonds in adulthood and demonstrate a shift in social preferences and relationships during juvenile development (Immelmann, 1972; Zann, 1996). Adult zebra finches are highly motivated to form pair bonds and remain paired, even when not actively breeding. The pair relationship is characterized by constant physical proximity, frequent vocal communication, and contact behaviors, such as clumping (perching in contact), allopreening (mutual grooming), and spending long periods of time together inside of a nest.

In recent years, there have been several studies that support the hypothesis that the nonapeptides play an important role in pair bonding in zebra finches. Two studies showed that antagonists acting primarily at the VT3 (OT-like) receptor increased the latency to pair and decreased pair formation in zebra finches, though these effects appear to be stronger in females than in males (Klatt & Goodson, 2013; Pedersen & Tomaszycki, 2012). Additionally, pairing for 48 hours increases expression of both AVT and MT in both the PVN and BSTm in both sexes (Lowrey & Tomaszycki, 2014). Consistent with this finding, experimental knockdown of endogenous MT production in the PVN significantly increased the latency to pair in females and reduced affiliative behaviors in zebra finches of both sexes (Kelly & Goodson, 2014a). Knockdown of AVT production in the PVN also reduces gregariousness in both sexes (Kelly & Goodson, 2014a). However, unlike prairie voles, partner preference is not induced by central infusions of either AVT or MT in adult zebra finches (Goodson et al., 2004).

The role of the extended medial amygdala

Although a number of brain regions appear to play a role in pair bonding, the extended medial amygdala, which includes both the medial portion of the amygdala (MeA) (nucleus taeniae (TnA) in birds) and the medial bed nucleus of the stria terminalis (BSTm), are important components of the circuit. Given their patterns of connectivity, neurons in the extended medial amygdala, which project to the lateral septum (LS), nucleus accumbens (NAcc), medial preoptic area (mPOA), and hippocampus (HP), are thought to play an important role in organizing appropriate responses to social stimuli (Goodson & Bass, 2001; Moore & Lowry, 1998; O'Connell & Hofmann, 2011).

AVP neurons in the extended medial amygdala are sensitive to social valence in both mammalian and avian species (Goodson et al., 2009; Goodson & Wang, 2006; Newman, 1999; Sheehan et al., 2001). Input from the MeA, both directly and via the BSTm, to the mPOA is critical for copulation in male rats (Kondo & Arai, 1995). Male hamsters with lesions of the MeA fail to successfully mate and show little chemoinvestigatory behavior with the female (Lehman & Winans, 1982; Lehman et al., 1980). The extended medial amygdala and nonapeptides appear to play an especially important role in the recognition of features of social partners, often in the olfactory domain (Young, 2002). Indeed, neurons in the MeA also show selectivity to stimulus odors from conspecifics of different sexes, as well as a striking sexual dimorphism in processing of olfactory information (Bergan et al., 2014). Oxytocin knockout mice fail to recognize the odor of familiar conspecifics and infusion of oxytocin into the MeA restores social recognition (Ferguson et al., 2001; Ferguson et al., 2000).

In several species of birds, there is an increase in the expression of c-Fos, an immediate early gene, in AVT-producing neurons in the BSTm in response to positively-valenced social stimuli,

including potential mating partners (Goodson et al., 2009; Goodson & Wang, 2006). Males that failed to reliably court females had fewer AVT neurons in the BSTm than did reliable courters and they failed to show an induction of c-Fos expression in response to exposure to a female (Goodson et al., 2009).

The specific contribution of the nonapeptides to the extended medial amygdala circuitry and the role these structures play in pair bonding is still unclear, even in mammals. AVP receptors are expressed in both the MeA and BSTm of the prairie vole and there is a higher binding density of V1a receptors (V1aR) in both the MeA and BSTm in monogamous prairie voles compared to montane voles (*Microtus montanus*) which do not form pair bonds (Insel et al., 1994; Phelps & Young, 2003). However, there is higher production of AVP in the MeA and BSTm in the non-monogamous vole species (Wang, 1995). Although there is higher immediate early gene activity in the MeA in paired versus unpaired prairie voles, administration of a V1aR antagonist does not block the formation of partner preference (Lim & Young, 2004). Confirming the functional role the MeA plays in pairing, lesions of the MeA result in reduced affiliative behavior in male prairie voles (Kirkpatrick et al., 1994). Furthermore, cohabitation with a female dramatically increases the number of cells in the BSTm that express AVP (Wang et al., 1994). These results suggest that the MeA and the BSTm are involved in affiliative behaviors and pair bonding, but a causal role for the nonapeptides in pairing has not been firmly established.

There is also limited evidence that neurons in the extended medial amygdala maybe involved in the formation and maintenance of pair bonds in birds. In male Japanese quail, lesions of the TnA reduce the expression of several behaviors associated with sexual interest in females (Thompson et al., 1998). In female zebra finches, there is a positive correlation between

immediate early gene activity in the TnA and the frequency of clumping and allopreening initiated by these females (Svec et al., 2009).

The development of pairing and affiliative behavior

The vast majority of research on the role of nonapeptides in pairing behavior has focused on the acute effects of these hormones in adults. However, there are several reasons why the neurobiology of affiliation should be studied from a developmental perspective. First, attachment to parents is observed very early in development in a wide range of species, suggesting that the neural substrate underlying affiliative behavior is established long before the formation of adult pair bonds (Ainsworth, 1989; Bowlby, 1960). Second, across species, early experience with caregivers during development is known to influence adult relationships, suggesting that these systems are plastic and sensitive to experience (Ainsworth, 1989; Bowlby, 1960; Champagne et al., 2003; Zayas et al., 2011). Third, from a broader perspective, a deeper understanding of the mechanisms involved in the development of affiliative behaviors is critical for our understanding of how evolution alters developmental processes to create novel behavioral phenotypes (Hofmann, 2010; Toth & Robinson, 2007).

Despite the evidence that nonapeptides play an important role in mediating social relationships across taxa in adulthood, there is not yet a compelling story regarding their role in the development of social behaviors in any species (Cushing, 2013). Work in both juvenile and adult rats suggests that manipulations of the AVP system during development can profoundly affect social behaviors (Boer et al., 1994; Boer, 1985; Bredewold et al., 2014; Schank, 2009; Veenema, Bredewold, & De Vries, 2012; Winslow & Insel, 1993). In prairie voles, injections of OT or OT antagonists on postnatal day 1 lead to changes in nonapeptide binding in several brain

regions in adults, but the directions of these changes are age-dependent, site-specific, and sexually dimorphic (Bales et al., 2007; Yamamoto et al., 2004). Prairie voles of both sexes exposed to OT on postnatal day 1 appeared to show a facilitation of pair formation and partner preference as adults (Bales & Carter, 2003a; 2003b) . Neonatal exposure to AVP increased aggression in adult male prairie voles (Stribley & Carter, 1999).

Previously, we demonstrated that intracranial injections of AVT early in development increased affiliative interest for parents in juvenile males, which was sustained throughout development (Baran et al., in review). In contrast, MC males did not show an affiliative interest in parents at any point in development and also did not show the normal increase in affiliative interest in females as they matured. Given these impacts on early attachment behaviors, we predicted that the effects of nonapeptide system manipulations on affiliative behaviors in juvenile zebra finches would extend into adult pairing relationships.

In this experiment, we tested the hypothesis that the nonapeptide arginine vasotocin (AVT, avian homologue of vasopressin) and a nonapeptide antagonist exert organizational effects on the neural pathways necessary for species-typical affiliative behavior in zebra finches. Organizational effects of a hormone occur early in development, when they establish the neural and physiological substrate for future behavior (Phoenix et al., 1959). Thus, we asked whether exogenous administration of AVT or an AVT antagonist early in development would alter the latency to form a pair bond and the expression of pair maintenance behaviors and the response to mate separation and reunion in adulthood. Second, do these manipulations permanently alter the adult distribution of neurons expressing both V1a receptor subtype (V1aR) and the extended medial amygdala (TnA and BSTm)? Finally, are the neurons that express V1aR mRNA the same as those activated in response to mate separation followed by reunion?

To test the organizational hypothesis, we manipulated the nonapeptide system of zebra finch chicks on days 2-8 post-hatching via daily intracranial (IC) injections of either AVT, Manning Compound (MC, a potent V1aR antagonist/mild OT receptor antagonist) or saline (vehicle control) and assessed the effects on pairing behaviors in adulthood, the expression of V1aR mRNA and the immunoreactivity of the immediate early gene, ZENK (Egr-1, a marker of neural activation) in the extended medial amygdala. We predicted that injections of AVT early in development would lead to increased sensitization to AVT later in development, which would lead to higher number of cells of expressing V1aR in the TnA and BSTm. Given the anatomical evidence for substantial growth and development of the TnA during the first 8 days post-hatching (Ikebuchi et al., 2013), we predicted that the treatment would have a particularly dramatic impact on the organization of this structure in adulthood. Furthermore, we predicted that increased sensitivity to AVT would lead to more rapid pair formation relative to Controls and an increase in affiliative behaviors directed towards a specific individual. We predicted that AVT antagonist would have the opposite effect, decreasing V1aR density and decreasing affiliative behaviors and the speed of pair formation relative to Controls.

Methods:

Breeding Conditions

Seventy-two unpaired adult males and females (hereafter "parents") were assigned to one of six breeding aviaries (1.2 x 0.9 x 0.6 m) and allowed to pair and breed. Offspring hatched within 40 days became the experimental subjects used in the study. Until approximately 40 days of age, subjects were cared for by the parents, which were provided with *ad libitum* access to finch seed, cuttlebone, grit, water, and supplemented weekly with hard-boiled egg. Parent pairs and nest box

occupancy were determined by observations of pair maintenance behaviors, including clumping, allopreening, and the occupancy of a nest box together. Observations were performed multiple times across several weeks by independent observers until pairing status was confirmed.

We checked the nests daily between 9:00 and 11:00 and the number of eggs, number of chicks, and chick mass were recorded. After 40-45 days of age, subjects were removed from their natal aviary and housed in same-sex aviaries in separate room from the parents. Each same-sex aviary contained birds of the same treatment to control for possible social interactions between adult birds in different treatments. All same-sex aviaries were in a single room, with a cage of adult females hidden behind a curtain to provide ambient colony noise.

Intracranial Injections

From day 2 through day 8 post-hatching, subjects received daily 2µL intracranial injections of either 1) AVT (10ng, (Arg8)-Vasotocin, Bachem 1785.0005); 2) Manning Compound (MC), a potent V1a and mild OT receptor antagonist (50ng, d(CH2)51,Tyr(Me)2,Arg8)-Vasopressin, Bachem 5350.0005); or 3) 0.9% isotonic saline (Castagna et al., 1998; Goodson et al., 2004; Manning et al., 1989). Both AVT and MC are predicted to act at multiple receptor subtypes in the zebra finch brain, including the VT4 (V1aR), VT3 (OT-like), and V2 receptors (Busnelli et al., 2013; Kruszynski et al., 1980; Leung et al., 2009; Manning et al., 2012).

IC injections were performed using a sterile 31G stainless steel insulin syringe, similar to Bender & Veney (2008). This technique is feasible because the zebra finch hatchling skull is thin, flexible, and easily penetrable by a needle. The chicks behave normally immediately following injections, including normal begging and locomotor behavior.

Chicks of each sex were randomly assigned to a treatment group on post-hatching day two, following genetic sexing. Chicks within the same clutch were randomly assigned to different treatment groups, such that treatment was unrelated to hatching order. Zebra finches typically become independent of parental feeding at 40 days post-hatching and reach sexual maturity between 60 and 90 days of age (Zann, 1996). Thus, after approximately 40 days of age (39.8 ± 5.4 days), subjects were removed from their natal aviary and housed in same-sex aviaries in a separate room from the parents. Each same-sex aviary contained birds of the same treatment to control for possible social interactions between birds in different treatments.

Pairing

In order to measure affiliative behavior and courtship in adulthood, subjects were randomly assigned an unmanipulated pair partner of the opposite sex (hereafter 'partner,' regardless of pair status) on day 90 post-hatch. Partners were drawn from a population of unpaired birds 6-12 months old. All partners had been housed in same-sex aviaries and rooms since 45-50 days of age and thus were sexually-naive and unpaired.

All introductions between the subjects and their pair partner took place in a small aviary (57 x 32 x 42 cm) in a room with no other birds in it in order to obtain high-quality recordings of courtship song for another experiment. After the introductions (lasting 15 to 45 minutes), the pair was immediately moved into a pair aviary in a colony room. Each aviary (57 x 32 x 42 cm or 61 x 36 x 43 cm) was provided with *ad libitum* access to finch seed, cuttlebone, grit, and water. However, because we were interested in pairing behaviors—not breeding—pairs were not given access to nest boxes or nesting material. The pairing aviaries were arranged in a large bank of aviaries, with white poster board separating each of the aviaries. This allowed for the pairs to be

visually but not acoustically isolated from the other pairs in the room. This allowed for ambient colony noise, but did not create competition for mates.

Pairs were then filmed in their aviaries for 16 minutes on each of days 2, 3, 4, and 6 between 12:00 and 18:00. All videos were scored by a trained coder who was blind to treatment for the duration of time spent perched in contact (clumping) and allopreening (both by the subject and by their partner). As non-social measures of general activity and feeding behaviors, we also measured the duration of time spent with the head in the food dispenser (including if the bird was sitting or standing inside the dispenser or if perched on the edge with the head inside the dispenser), the number of visits to the food dispenser, and the number of visits to the water dispenser.

Mate Separation, Reunion and Euthanasia

In order to induce pairing behaviors, on the seventh day following introduction subjects were separated briefly from their pair partner. The partner was removed from the cage for approximately one hour and moved temporarily to the same-sex social aviary in which they were housed prior to pairing, which was located in another room. Previous studies have shown that a separation of this length induces a stress response, as well as leads to a marked increase in affiliative behaviors upon reunion (Prior et al., 2013; Prior et al., 2014; Remage-Healey et al., 2003). After one hour of separation, the partner was returned to the pair aviary and the reunion was filmed for 25 minutes. Videos were again scored by a trained coder who was blind to treatment for the same measures as above, plus additionally the number of song bouts performed by male subjects.

Following an additional 55-65 minutes in the pair cage (which was not filmed), the subjects were euthanized via rapid decapitation. Thus, euthanasia occurred 90 minutes after reunion with the partner. The number of birds that completed treatment and survived until the completion of the study are as follows: Control males (N = 7); AVT males (N = 11); MC males (N = 11); Control females (N = 10); AVT females (N = 9); and MC females (N = 9).

Tissue Collection and Sectioning

Following euthanasia via rapid decapitation, the brain was immediately extracted (under 5 min) and frozen in cold methylbutane and stored at -80C until sectioning. Brains were sectioned coronally into six series at 20µm on a Leica cryostat and mounted directly onto SuperFrost Plus slides (Fisher Scientific, Pittsburgh, PA).

Probe Preparation

The V1aR probes were derived from published sequences (Genbank: V1aR = XM_002187285). We developed primers for the V1a receptor using the NCBI primer tool (V1aR: Forward = AGCGCGGCTCGCAAGTCTAC; Reverse = GAAGGGCGCCCAGCAAACGA, beginning at 700bp of the published sequence), and conducted reverse-transcribed PCR (#12574-035, Invitrogen, Carlsbad, CA) per manufacturer's instructions on isolated RNA to obtain the cDNA. The resulting product was sequenced using the Applied Genomics Technology Center (AGTC) at Wayne State University. Product size for V1aR (344bp) was confirmed. Sequence identity for zebra finch V1aR was also confirmed using the BLASTn tool on the NCBI website. The probe was prepared using a Roche Applied Science

DIG RNA Labeling kit according to manufacturer's instructions (catalog # 11175025910, Indianapolis, IN).

Dot Blot Assay

To determine the ideal probe concentration, we performed a dot blot assay as in (Lowrey & Tomaszycki, 2014; Patel et al., 2012). Briefly, the probes were serially diluted and spotted onto a charged nylon membrane (Millipore, Billerica, MA, USA), incubated in a secondary antibody (1:1500, alkaline phosphatase conjugated anti-digoxigenin, Roche, Indianapolis, IN), and visualized using NBT/BCIP (Roche Applied Science, Indianapolis, IN). The lowest concentration that yielded a detectable spot (1:1000) was then used for *in situ* hybridization. We have confirmed that this method results in highly specific and high quality staining with no detectable background (Lowrey & Tomaszycki, 2014).

Immunocytochemistry and In Situ Hybridization

Fluorescence immunocytochemistry and *in situ* hybridization was then conducted using a protocol adapted from previous work (Wu et al., 2010). Slides were fixed with 3% paraformaldehyde, acetylated, dehydrated, and air dried. Hybridization occurred at 55°C overnight. After a series of washes, slides were incubated in 0.3% hydrogen peroxide in Tris-NaCl-Tween (TNT) buffer for 10 minutes, and blocked in TNT buffer with 2mg/ml of bovine serum albumin (TNB) for 30 min. Slides were then incubated in the secondary antibody (1:100, Anti-DIG-POD, #11207733910, Roche Applied Science, Indianapolis, IN) for 2hr, followed by 30 minutes in a tyramide-conjugated fluorophore (1:100, Alexa 488, Invitrogen, Carlsbad, CA). Antigen retrieval was accomplished using a 10mM Sodium Citrate Buffer heated to 70°C for 30

min. Slides were then processed for ZENK immunocytochemistry. Each step was preceded by 3 washes (5 min each) in Tris Buffered Saline (TBS). Slides were blocked in 2% Normal Goat Serum and 0.3% Triton X-100 for 30 minutes. This was immediately followed by a 48 hour incubation in the ZENK primary antibody (1:1000; cat. # sc189; Santa Cruz Biotechnology, Santa Cruz, CA, USA) at 4°C. Slides were then incubated for 2 hour in the secondary antibody, which was conjugated to a fluorophore (5 µl/ml; goat anti-rabbit secondary conjugated to Alexa 594, Invitrogen, Carlsbad, CA). Finally, slides were cover-slipped with Slow Fade Antifade Gold with DAPI (#S36938, Invitrogen, Carlsbad, CA) and sealed with nail polish. V1aR sense slides exhibited no staining, and staining in the anti-sense slides appeared to have a distribution similar to that reported in earlier studies (Leung et al., 2011). All slides were stained at the same time, to control for potential differences in staining quality.

Quantification

Slides were analyzed using a Leica DM 5500B microscope and Leica Application Suite IX. An observer (NMB), who was blind to treatment, quantified the number of cells in a 400 X 400 µm counting frame (by hand, using the Image J Cell Counter plugin to keep track of cell counts) in each region. For ZENK-ir, only red cells exhibiting a punctate nuclear staining were quantified. Regions of interest were located using adjacent sections stained with thionin and the Nixdorf-Bergweiler and Bischof zebra finch atlas. To account for the proportion of cells in our counting frame, we counted the number of DAPI+ nuclei using the particle analysis in Image J. For the TnA, 4 ± 2 counting frames taken from 3 separate sections (one anterior, one intermediate, and one posterior) representing both left and right hemispheres were quantified per animal. For the BSTm, 3 ± 1 counting frames taken from 2 separate sections [one ventral to the

anterior commissure (AC) where the AC is strongest and the second where the AC just begins to disappear and the BSTm cells appear to spill over the occipito-mesencephalic tract (OM)]. For each counting frame, three measurements were taken: the number of cells immunoreactive for ZENK (ZENK-ir), the number of cells expressing V1aR, and the number of cells expressing both. Due to tissue quality, one Control male, two AVT males, and four MC males were excluded from the analysis, for a final sample size of 6 Control males, 9 AVT males, and 7 MC males.

Statistical Analysis

All statistical analyses were performed with R software (R Development Core Team 2007). We used a survival analysis to test whether treatment affected the time until the first pair maintenance behavior was observed (clumping or allopreening by either individual in the pair). We used the *survfit* function in the survival package, which computes the predicted survivor function for a Cox proportional hazards model in dependence of specified values of the predictor variable(s) in the model (in this case, treatment), followed by a log-rank test using the *survdiff* function (Fox & Weisberg, 2011; Therneau, 2015; Therneau & Grambsch, 2000). To test if treatment affected whether or not an individual formed a pair, we used a general linear model (GLM) with a binomial link function. To test the effect of the treatment on the amount of clumping, allopreening, time spent in the food dispenser, visits to the food dispenser, and visits to the water dispenser across test days, we used a linear mixed model (LMM). In this model, Sex, Treatment, and Test Day (Day 2, 3, 4, 6, and Reunion) were specified as fixed factors. Random factors were individual ID nested within Family ID. The interaction effect considered was Sex*Treatment*Test. We then performed a similar LMM within each sex. To test the

significance of each fixed effect within a model, we used the Kenward-Roger approximation to get approximate degrees of freedom and the t-distribution to get p-values (Kenward-Roger in the pbkrtest package). We used a Poisson GLM to test the effect of treatment on the number of song bouts performed by males during the reunion.

To analyze cell count data, we averaged the cell counts within regions and within subjects and rounded the counts to the nearest integer. We then analyzed the cell counts using weighted negative binomial GLMs using the *glm.nb* function in the MASS package, with the number of sections contributing to the averaged count included as the weight. This method is preferable for several reasons. First, there is an emerging consensus that Poisson GLMs are nearly always preferable to log-transforming count data (O'Hara & Kotze, 2010). Furthermore, negative binomial regression can be used for over-dispersed count data (i.e., when the conditional variance exceeds the conditional mean) and there was evidence of over-dispersion in this data set. Negative binomial GLM is a generalization of Poisson regression since it has the same mean structure as Poisson regression and it has an extra parameter to model the over-dispersion. Finally, we used the weights to ensure that counts that were obtained from a larger number of sections were more heavily weighted in the analyses, since we can be more confident that these counts represent the true mean within a given individual and brain region.

To perform model comparisons for the GLM, LMM, and negative binomial GLM models, we used likelihood ratio tests to compare the full model to a reduced null model with only the factor of interest removed using the *anova* function to perform a chi-square test. In addition, we performed post hoc tests on the interaction terms using the *testInteractions* function in the phia package.

Results:

Pairing Behavior

There was no effect of treatment on whether subjects formed a pair in either sex (GLM: Males, $X^2(2) = 4.2$, $p = 0.1$; Females, $X^2(2) = 1.6$, $p = 0.4$). There was no evidence that treatment affected the time to pairing (i.e., the number of days until first observed instance of either perching in contact or allopreening) in either females (Log-rank test: $X^2(2) = 2.6$, $p = 0.3$, Figure 11a) or males (Log-rank test: $X^2(2) = 0.7$, $p = 0.71$, Fig. 1b).

However, a full model predicting the proportion of time spent clumping including treatment, sex, and test day as factors was nearly significant ($X^2(8) = 15.5$, $p = 0.05$) (Figure 12a and 2b). Within females, treatment was not a significant predictor of the proportion of the test time spent perched in contact ($X^2(2) = 2.2$, $p = 0.3$) (Figure 12a). However, the proportion of time spent perched in contact differed across test days ($X^2(4) = 19.5$, $p = 0.0006$). Consistent with previous mate-separation and reunion paradigms, the proportion of time spent clumping was significantly higher during reunion than during observations on previous days ($t = 2.4$, $p = 0.02$).

Within males, there was a significant interaction between treatment and day in the proportion of time during the tests spent perched in contact with their partner ($X^2(8) = 26.3$, $p = 0.0009$) (Figure 12b). Similar to what was observed in females, there was a significant effect of test day, with a higher proportion of clumping observed during the reunion than on previous test days ($t = 2.5$, $p = 0.02$). In addition, AVT males spent a significantly greater proportion of time clumping with their partner during the reunion test than Control males ($t = 3.2$, $p = 0.002$). Within males, the effect of treatment on the time perched in contact with the female partner during the 25 minute reunion was significant ($X^2(2) = 15.7$, $p = 0.0004$) (Figure 14a). AVT males spent, on average, 45% of the 25 minute reunion period perched in contact with their female partner.

Indeed, AVT males spent 790% more time clumping than Control males ($X^2(1) = 17.5$, $p < 0.0001$) and 360% more time than MC males ($X^2(1) = 14.0$, $p = 0.0004$), whereas there was no difference between MC and Control males ($X^2(1) = 0.4$, $p = 0.5$).

In addition, we tested whether or not there were effects of treatment, sex, and test day on the time that the subject spent allopreening with their partner. There was no significant effect of either treatment or sex, but there was a significant effect of test day ($X^2(4) = 27.6$, $p <0.0001$) in both females (Figure 13a) and males (Figure 13b). Again, there was a significant increase in the proportion of time that the subject was observed allopreening their partner during the reunion, compared to the other test days ($t = 4.0$, $p = 0.0007$). There was not a significant effect of any of the variables on the proportion of time that the partner was observed allopreening the subject.

In addition, there was a significant effect of treatment on the number of song bouts during the reunion (Poisson GLM: $X^2(2) = 80.1$, $p < 0.0001$) (Figure 14b). In this case, each treatment group was significantly different from each of the others (Control-AVT: $Z = -8.7$, $p < 0.0001$; Control-MC: $Z = -3.2$, $p = 0.003$; and AVT-MC: $Z = 5.7$, $p < 0.0001$).

As a control, we tested whether or not there was a significant effect of treatment on either feeding or drinking behavior, which might be expected with manipulations of the AVT system. In addition, group differences in general activity level could be associated with differences between pairing behaviors. However, there was no significant effect of treatment on the proportion of time spent with the head in the food dish or number of visits to the food dish, controlling for both sex and test day (Proportion: $X^2(2) = 0.1$, $p = 0.9$; Number of visits: $X^2(2) = 2.6$, $p = 0.3$). There was also no significant effect of treatment on the number of drinks of water consumed by subjects across test days ($X^2(2) = 4.5$, $p = 0.1$).

Effect of treatment on V1aR expression and the immediate early gene ZENK

Because the only treatment effects on behavior were observed in males, we analyzed whether early injections of AVT or MC impacted the expression of V1aR and an immediate early gene reflecting neuronal activity in the extended medial amygdala (BSTm and TnA) in male subjects only. There were significantly more V1aR-expressing cells per counting frame in the BSTm (199 ± 51 cells) compared to the TnA (82 ± 33 cells) (nbGLM: $X^2(1) = 180.5$ $p < 0.0001$). There was no significant difference in the number of ZENK-ir cells between the two regions (BSTm: 29 ± 27; TnA: 32 ± 20) (nbGLM: $X^2(1) = 0.2$, $p = 0.7$). See Figure 15 for example staining.

Treatment did not predict the number of V1aR-expressing cells in the BSTm (nbGLM: $X^2(2) = 1.9$, p = 0.4) (Figure 16a, first panel). However, treatment was a significant predictor of the number of V1aR-expressing cells in the TnA (nbGLM: $X^2(2) = 34.6$, $p < 0.0001$) (Figure 16a, second panel). In this case, AVT males were found to have a significantly higher expression of V1aR in the TnA than both Control and MC males (Control-AVT: $X^2(1) = 21.9$, $p < 0.0001$; Control-MC: $X^2(1) = 0.2$, $p = 0.6$; AVT-MC: $X^2(1) = 32.8$, $p < 0.0001$).

Treatment was also a significant predictor of the number of ZENK-ir cells in the BSTm (nbGLM: $X^2(2) = 11.8$, $p = 0.003$) (Figure 16b, first panel). MC males had lower numbers of ZENK-ir cells in the BSTm compared to AVT males (Control-AVT: $X^2(1) = 6.5$, $p = 0.02$; Control-MC: $X^2(1) = 0.3$, $p = 0.6$; AVT-MC: $X^2(1) = 11.7$, $p = 0.002$). In addition, treatment was a significant predictor of the ZENK-ir cells in the TnA (nbGLM: $X^2(2) = 5.8$, $p = 0.05$) (Figure 16b, second panel). AVT males were found to have lower numbers of ZENK-ir cells in the TnA compared to MC males (Control-AVT: $X^2(1) = 0.1$, $p = 0.7$; Control-MC: $X^2(1) = 2.8$, $p = 0.18$; AVT-MC: $X^2(1) = 5.8$, $p = 0.05$). ZENK-ir in the TnA was positively correlated with the time the subject spent perched in contact with his female partner (nbGLM: $X^2(1) = 5.6$, $p = 0.02$). We also

observed a slight but significant negative correlation between singing and ZENK-ir in the BSTm (nbGLM: $X^2(1) = 5.7$, $p = 0.02$).

Treatment also predicted the co-localization of V1aR and ZENK in the BSTm (nbGLM: $X^2(2) = 12.4$, $p = 0.002$) (Figure 17a, first panel). AVT males had significantly fewer cells with both V1aR and ZENK in the BSTm compared to both MC and Control males (Control-AVT: $X^2(1) = 5.7$, $p = 0.03$; Control-MC: $X^2(1) = 0.7$, $p = 0.4$; AVT-MC: $X^2(1) = 13.1$, $p = 0.0009$). Additionally, treatment significantly predicted the number of V1aR+ZENK cells in the TnA (nbGLM: $X^2(2) = 8.1$, $p = 0.02$) (Figure 17a, second panel). AVT males had a greater number of cells co-localized for both V1aR and ZENK than MC males (Control-AVT: $X^2(1) = 3.4$, $p = 0.1$; Control-MC: $X^2(1) = 0.3$, $p = 0.5$; AVT-MC: $X^2(1) = 7.2$, $p = 0.02$).

There was no significant effect of treatment on the total number of cells per counting frame (i.e., the number of DAPI-stained nuclei) (nbGLM, BSTm: $X^2(2) = 0.4$, $p = 0.8$; TnA: $X^2(2) = 5.4$, $p = 0.07$) (Figure 17b). This suggests that treatment did not impact the overall number of cells in these regions.

In addition, there were significant correlations between the number of V1aR+ZENK cells and behaviors exhibited by males during the reunion. In the BSTm, there was a negative relationship between the number of V1aR+ZENK cells and clumping behavior (nbGLM: $X^2(1) = 8.0$, $p = 0.005$) (Figure 18a), but was no significant relationship between V1aR+ZENK co-localization and singing behavior (nbGLM: $X^2(1) = 1.4$, $p = 0.2$) (Figure 18b). In the TnA, there was a strong positive relationship between V1aR+ZENK co-localization and clumping behavior (nbGLM: $X^2(1) = 11.5$, $p = 0.0007$) (Figure 19a) and was a strong negative relationship between V1aR+ZENK co-localization and the number of song bouts (nbGLM: $X^2(1) = 9.9$, $p = 0.002$) (Figure 19b).

Discussion:

These results provide the first evidence that AVT and nonapeptide receptors are involved in the development of affiliation and pair maintenance behaviors in adult birds. In this experiment, Control males were characterized by high numbers of song bouts and very modest amounts of affiliative behaviors. In contrast, AVT males sang substantially less than Controls, but were characterized by remarkably high rates of affiliative behaviors, perching in contact with their partner nearly eight times as much as Control males following reunion with their partner. In AVT males, we also found higher expression levels of V1aR and higher number of cells co-localized for V1aR+ZENK in the TnA. MC males, on the other hand, sang less, perched in contact less with their partner, and had reduced ZENK expression in the BSTm. We also demonstrated an association between the co-localization of V1aR and ZENK in the extended medial amygdala (TnA and BSTm) and both affiliation and amount of singing.

These results are broadly consistent with the now accumulating evidence that nonapeptides play an important role in pair bonding in birds. Indeed, the effects of early injections of AVT have a truly remarkable effect on affiliative behavior in adult males. The first experiments to investigate the role of adult nonapeptides on pairing did not uncover significant effects of central infusion of either AVT or AVT antagonists on affiliation, partner preference, or courtship singing (Goodson et al., 2004). However, the actions of nonapeptides are known to have site-specific and sometimes opposing effects on different brain regions (Goodson, 2005; Goodson et al., 2005; Newman, 1999). Of course, the MeA has strong bidirectional connections with the BSTm, but activity in each of these structures have frequently been associated with different behaviors, suggesting that they contribute in different ways to the expression of these behaviors (e.g. Kollack-Walker & Newman, 1995). Consistent with this literature, we find opposite

relationships between the ZENK-ir (and the co-localization of V1aR and ZENK) and clumping behavior across the two brain regions, the BSTm and TnA. Our data also suggest that manipulations of the AVT system early in life can have opposite effects on the organization of these two nuclei.

Additionally, these results suggest that affiliative behavior (a non-sexual, pair maintenance behavior) is both neurally and behaviorally dissociable from singing and courtship behaviors (more sexually-motivated behaviors). For example, we find that there is a positive correlation between affiliation with the pair partner and immediate early gene activity in the TnA, which is consistent with evidence that the MeA is involved in affiliative behavior between pair partners in both mammals and song birds (Kirkpatrick et al., 1994; Svec et al., 2009). However, the role that the TnA plays in pairing in adult zebra finches is still poorly understood, though it may be likely that it plays a similar role as in mammals, including individual recognition and contextually-appropriate social behaviors.

Furthermore, these are the first results to demonstrate that AVT and the V1aR receptor are important in pairing behavior in male zebra finches. Previous research on the role of nonapeptides in pairing in zebra finches had found significant effects of MT and OT-like receptors, mostly in females, but had not yet identified a role for V1aR in pairing in either sex (Kelly & Goodson, 2014a; Klatt & Goodson, 2013; Pedersen & Tomaszycki, 2012). Thus, the present findings are broadly consistent with decades of research in prairie voles demonstrating that AVT and V1aR are more important than OT and OTR for pairing behavior in males (Pitkow et al., 2001; Williams et al., 1992; Winslow et al., 1993).

However, another possible explanation for the lack of effects in females is that perhaps the intracranial injections occurred too early in development to substantially alter nonapeptide

circuitry. Developmental work in rodents suggests that the AVT system develops more rapidly in males, with measurable quantities of both the production of AVP and its receptors being observed much earlier in development in males compared to females (Buijs et al., 1980; Szot & Dorsa, 1993). If the AVT system of female zebra finches is similarly delayed, it is possible that intracranial injections on days 2-8 simply occurred before there were functional receptors expressed at high enough levels to have obvious organizational effects on brain and behavior. However, since there is no descriptive work outlining the development of the nonapeptide circuitry in birds (and we only assessed the effects on days 2-8 post-hatching), this hypothesis remains to be tested.

Previous work in zebra finches and other song birds has demonstrated that adult AVT plays a prominent role in both gregariousness and aggression, but not pairing, particularly when acting in the lateral septum (LS) (Kelly et al., 2011). In zebra finches, the LS appears to express a high density of binding of both AVT and OT, as well as high expression of OTR mRNA (Kelly et al., 2011; Leung et al., 2011, 2009). Infusions of MC in the LS reduces the preference to affiliate with larger flocks of birds and increases anxiety-like behaviors (Kelly et al., 2011). Although we do not present data for the LS here, there is evidence that MC males are perhaps more gregarious than Control or AVT males. Although we found no evidence that MC males failed to form pair bonds or had a longer latency to form these pairs, it is important to note that pairing in this experiment occurred in small pair aviaries, so the presence of affiliative behavior observed in the MC males may be a function of preferring to affiliate with other birds in general, rather than the tendency to form a specific pair bond with a female. In a previous study, these same MC males were found to spend more time than Control or AVT males in proximity with other birds in four-way affiliative preference tests throughout development, but showed no specific preference to

affiliate with female conspecifics (Baran et al., in review). Thus, future work could investigate the effects of these early life manipulations on more general gregariousness or sociality. The specificity of the affiliation observed in this experiment could be further assessed using something akin to a partner preference test. Additionally, a conditioned place preference paradigm might be used to determine whether these manipulations have altered the reward value of the pair partner versus familiar conspecifics generally.

We did not observe any effects of treatment on whether males appeared to form a pair bond, on the latency to form the pair, or on the expression of pair maintenance behaviors during the observations prior to separation and reunion. This is perhaps because we only observed pairs for relatively limited amounts of time each day, which may not have been enough time to capture group differences in affiliation, which normally occur at a relatively low frequency. The group differences may have been more evident during the reunion because, as expected, the separation from the partner induced a higher expression of affiliative behaviors.

Additionally, because pairing in this experiment took place in small aviaries, there was no opportunity to observe the effects of treatment on the competition between males for female partners. However, given the effect of treatment on courtship song (Chapter 5), we would predict substantial differences between treatments on these measures if males needed to compete with other males for the attention of a limited number of females. We also did not assess the effects of the manipulations to the AVT system on aggressive behaviors, which may also be particularly relevant in the competition for mates or access to limited resources such as nest sites.

Unlike in previous studies in zebra finches, we did not find a positive relationship between ZENK-ir in the BSTm and singing (Goodson et al., 2009). Instead, we observed a slight negative correlation between singing and both ZENK-ir in the BSTm, as well as between singing and

V1aR+ZENK in the BSTm and TnA. This may be because these samples were not collected from males courting novel females, but instead singing to females with whom they are already paired and in the context of re-establishing an already formed pair bond. Heimovics and Riters (2005, 2006) observed differential regulation of starling song by the BSTm depending upon the context in which it is produced. Given that these samples were collected after separation from the female partner, it is possible that song in this context functions as a pair maintenance behavior, rather than a courtship or sexual behavior. Indeed, no attempted or successful copulations were observed in any of the reunion periods, suggesting that song is not serving a courtship or sexual function in the reunion test.

Overall, these results demonstrate that manipulations of the AVT system during development can induce plasticity in the expression of V1aR in zebra finches. From a broader perspective, it is possible that developmental plasticity in the nonapeptide circuitry may in fact underlie the evolution of novel social phenotypes. We certainly know that the nonapeptides are among the most evolutionarily labile signaling systems in the vertebrate brain (Goodson, 2005; Goodson et al., 2005). Previous research has also demonstrated that there can be remarkable individual differences in receptor expression across brain regions within a species (Phelps & Young, 2003). Evidence from prairie voles also suggests that these systems can be altered developmentally (Bales & Carter, 2003; Bales et al., 2007; Stribley & Carter, 1999; Yamamoto et al., 2004; Yamamoto et al., 2006). Thus, these data add to the growing literature that provides support for the idea that variation in the nonapeptides and their receptors, as well as the developmental processes that construct the social brain, may support the evolution of the remarkable diversity of social phenotypes observed across taxa.

It is also worth pointing out that our effects appear to be quite specific to the social domain. We observed no effect of the treatment on either feeding or drinking behaviors (which are certainly possible given AVT's role in maintaining water balance. We also found no effects of these treatments on growth or survival in chicks or on activity level throughout development (Baran et al., in review). However, we cannot rule out the possibility that these treatments exerted widespread effects on the hypothalamic-pituitary-adrenal (HPA) axis. AVP/AVT serves as a releasing factor, along with corticotropin releasing factor (CRF), for the production of adrenocorticotropic hormone (ACTH) in the anterior pituitary (Buckingham, 2009). Previous work in our lab has shown that separation from the partner results in an elevation of corticosterone, which returns to baseline when individuals are reunited with their pair partner (Remage-Healey et al., 2003). However, there does not appear to be a buffering effect of being isolated with the pair partner (Banerjee & Adkins-Regan, 2011). Thus, while there does appear to be a relationship between the HPA axis and pairing, whether these observed behavioral effects are also partially mediated through alterations to the HPA axis remains to be tested.

Although previous studies have provided only modest evidence of V1aR expression in either the TnA or the BSTm in zebra finches (Leung et al., 2011, 2009), both of these regions do consistently express this receptor subtype in voles and there is evidence of AVT binding within these regions in zebra finches, white throated sparrows (*Zonotrichia albicollis*), and canaries (Leung et al., 2011, 2009; Voorhuis et al., 1988). We find that there is staining in these regions, but it is relatively low (only 15.1% of neurons in the TnA and 30.6% in the BSTm), with only a very small proportion of neurons exhibiting co-localization. These results also suggest that there is potential for plasticity in the expression of V1aR in neurons in the TnA and that the activity of these neurons is correlated with behavior, suggesting that the expression of nonapeptide

receptors may depend on developmental experiences, pairing status, or other aspects of social context.

Nevertheless, considering both the magnitude of the manipulation and the extent of the behavioral changes, these neural effects are, in fact, quite modest. Thus, it seems unlikely that the observed changes in the TnA and BSTm alone can account for the extreme changes in behavior observed in this study. Indeed, we would predict that such manipulations could result in quite profound and widespread alterations to a number of structures that were not investigated here, including regions known to contain high levels of V1aR in zebra finches. Thus, future research should investigate whether these manipulations have resulted in changes in the expression of V1aR in other brain regions, as well as in the production of AVT itself.

Conclusion:

These results are the first demonstration that the nonapeptide system may function early in development to influence adult social behaviors in birds. Males in each of the three treatment groups exhibited dramatically different profiles in terms of their courtship and pairing behaviors, and yet they all appeared to form pair bonds. This suggests that the nonapeptides could underlie the differentiation between different mating "strategies" in other socially monogamous species where such strategies have been found (e.g. McGlothlin et al., 2007). Some recent evidence suggests that individual variation in nonapeptide receptor expression in the brain predicts variation in mating tactics in prairie voles (Berrio et al., 2014; Ophir et al., 2012; Ophir et al., 2008). Coupled with the increasing evidence that early social experiences shape adult social relationships and the social brain, it is possible that the nonapeptide actions are an important process in this developmental pathway.

In this experiment, we employed a relatively crude first-pass approach to determine whether the nonapeptides play an organizational role in the social brain by injecting relatively high doses of either AVT or MC into the brains of developing songbirds. The ability to interpret these effects would be greatly enhanced by descriptions of the development of the nonapeptide system in zebra finches. Furthermore, future studies with better spatiotemporal precision will help to elucidate the specific functions of nonapeptides in specific brain regions during development. This would allow researchers to understand how these circuits are involved in the development of social behaviors, both adaptive and maladaptive. Nevertheless, these results provide strong evidence that the nonapeptides play a critical role in social development and that changes to this system during development can have a profound effect on the social brain throughout life.

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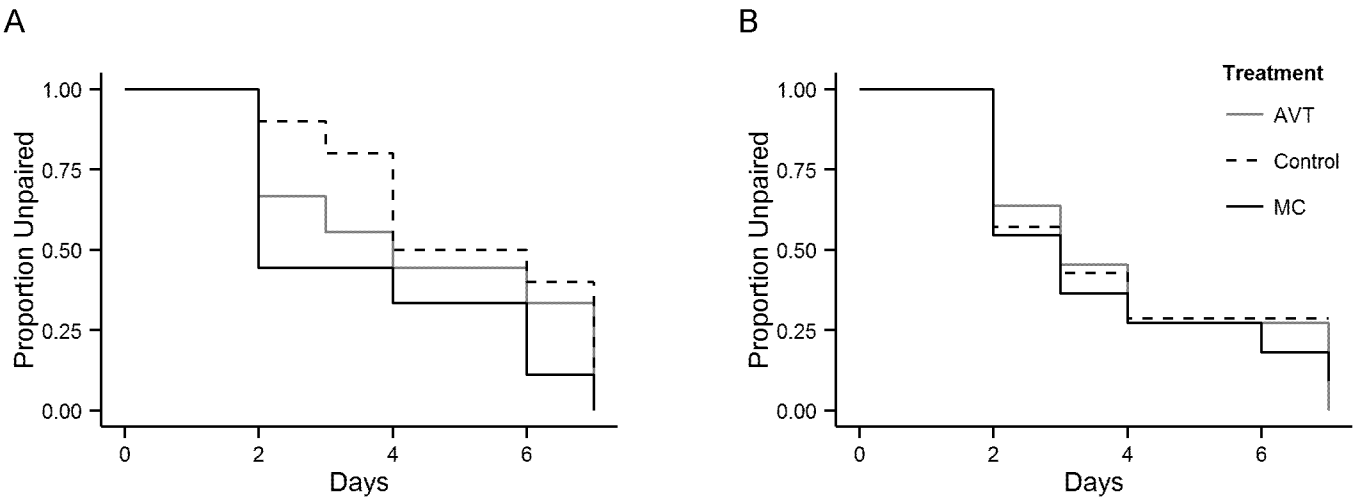

Figure 11: Survival plot of the proportion of unpaired subjects by treatment

The proportion of individuals who had not been observed either perching in contact or allopreening by test day for A) female subjects and B) male subjects. Subjects received intracranial injections on post-hatch days 2-8 of either arginine vasotocin (AVT), Manning Compound (MC, a V1aR antagonist), or vehicle control of saline (Control). Control subjects are depicted with a solid gray line, AVT with a dashed black line, and MC with a solid black line.

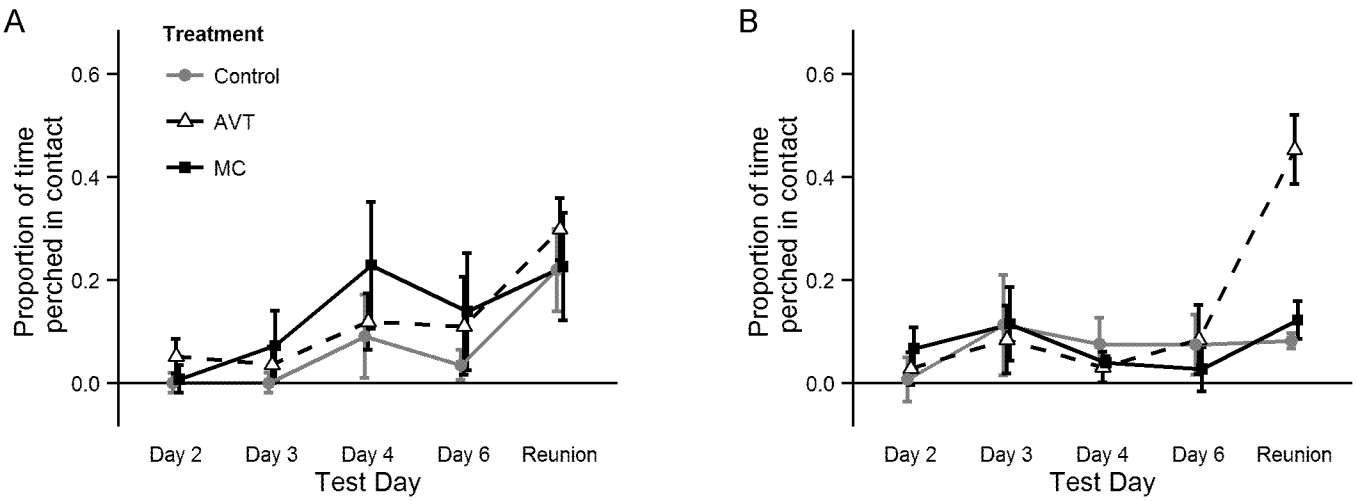

Figure 12: Proportion of time perching in contact with the partner across test days

Mean ± SE of the proportion of time spent perching in contact (clumping) with the partner by test day for A) females and B) males. Subjects received intracranial injections on post-hatch days 2-8 of either arginine vasotocin (AVT), Manning Compound (MC, a V1aR antagonist), or vehicle control of saline (Control). Control subjects are depicted with circles and a solid gray line, AVT with triangles and dashed black line, and MC with squares and solid black line.

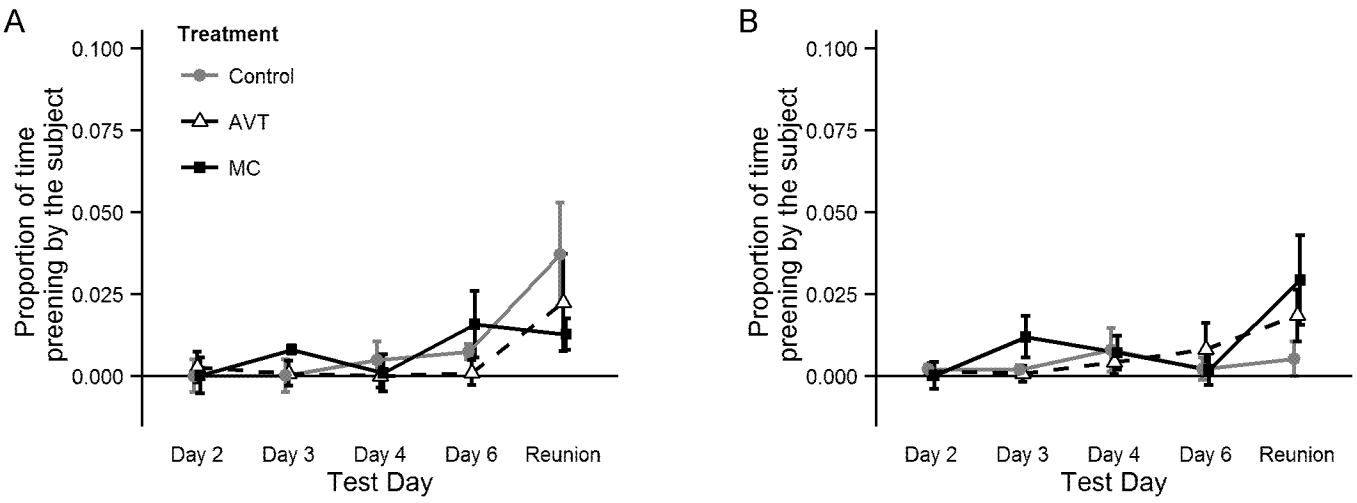

Figure 13: Proportion of time spent allopreening by the subject across test days

Mean ± SE of the proportion of time spent allopreening, or mutual grooming, by the subject across test days for A) females and B) males. Subjects received intracranial injections on post-hatch days 2-8 of either arginine vasotocin (AVT), Manning Compound (MC, a V1aR antagonist), or vehicle control of saline (Control). Control subjects are depicted with circles and a solid gray line, AVT with triangles and dashed black line, and MC with squares and solid black line.

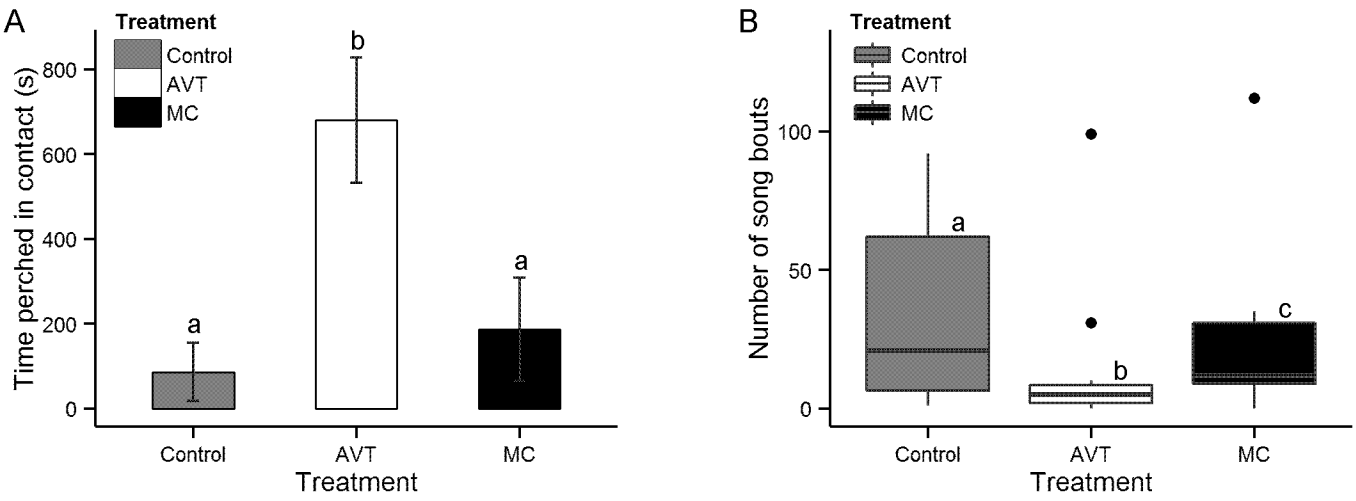

Figure 14: Time perched in contact and number of song bouts by male subjects

A) Mean ± SE time (s) spent perched in contact with the female partner during the 25 minute reunion period following a one-hour separation. B) Box-and-whisker plot of the number of song bouts during the first 25 minutes following reunion with the female partner after a one-hour separation. Data beyond the ends of the whiskers are outliers and plotted as points. Subjects received intracranial injections on post-hatch days 2-8 of either arginine vasotocin (AVT), Manning Compound (MC, a V1aR antagonist), or vehicle control of saline (Control). Letters indicate groups that are significantly different from each other.

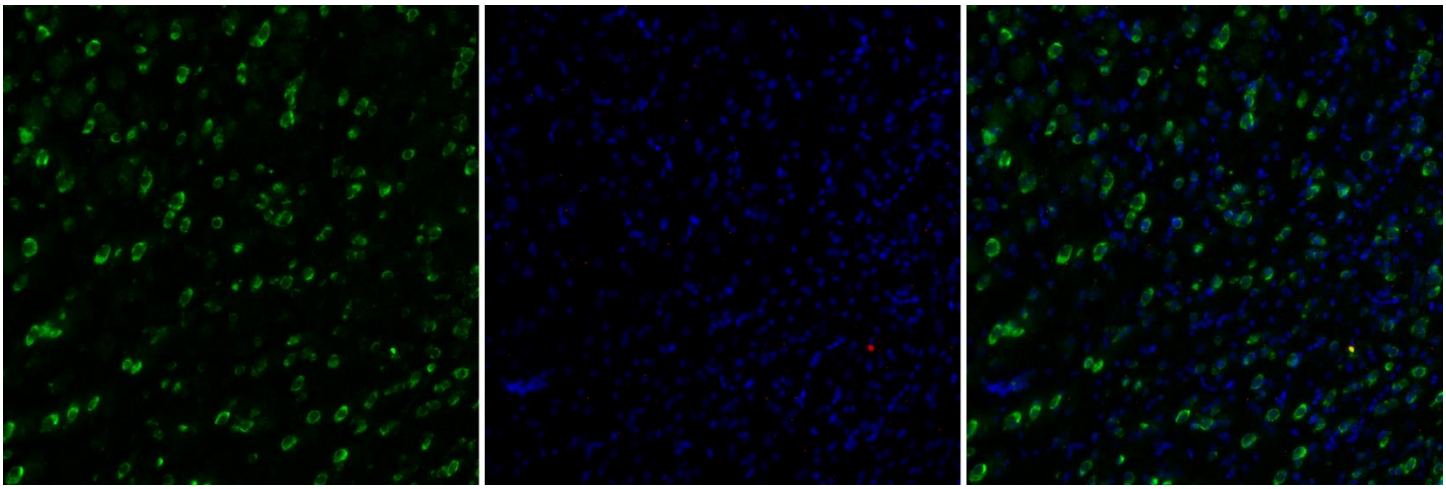

Figure 15: Example double-label fluorescence *in situ* staining of the TnA in a Control male

In the first panel, V1aR expressing cells are labeled in green. Punctate nuclear-staining for ZENK is shown in red, with DAPI nuclear stain shown in blue. The third panel shows all three—V1aR, ZENK, and DAPI—combined. The frame is 400 x 400 microns.

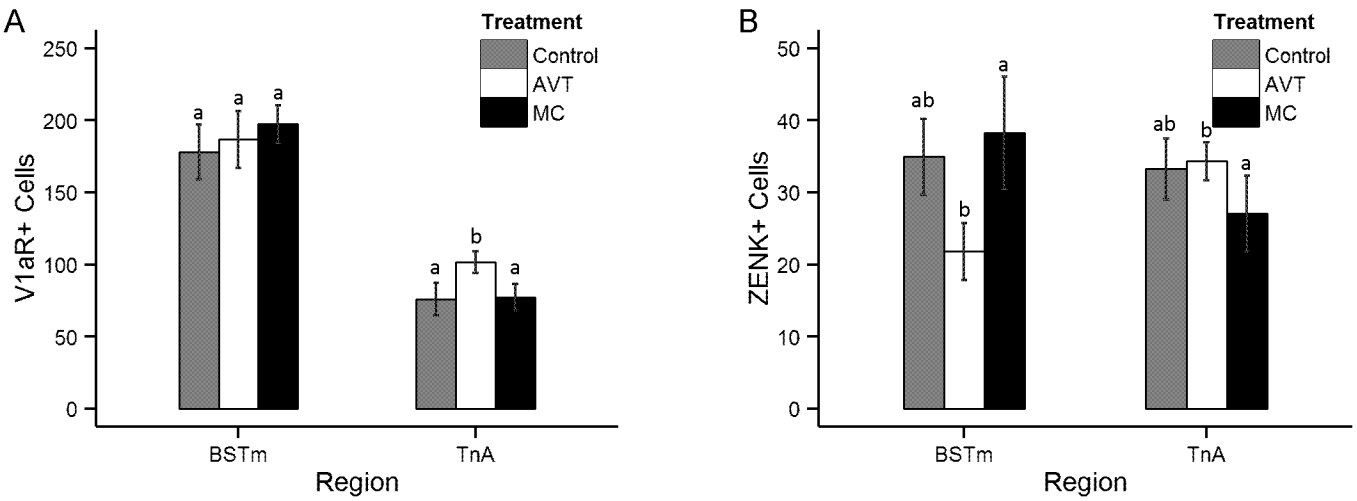

Figure 16: Number of V1aR and ZENK expressing cells in the BSTm and TnA

A) Mean ± SE number of cells expressing V1aR in the bed nucleus of the stria terminalis (BSTm) and the nucleus taeniae (TnA). B) Mean ± SE number of cells immunopositive for ZENK (Egr-1) in the BSTm and the TnA. Subjects received intracranial injections on post-hatch days 2-8 of either arginine vasotocin (AVT), Manning Compound (MC, a V1aR antagonist), or vehicle control of saline (Control). Letters indicate groups that are significantly different from each other.

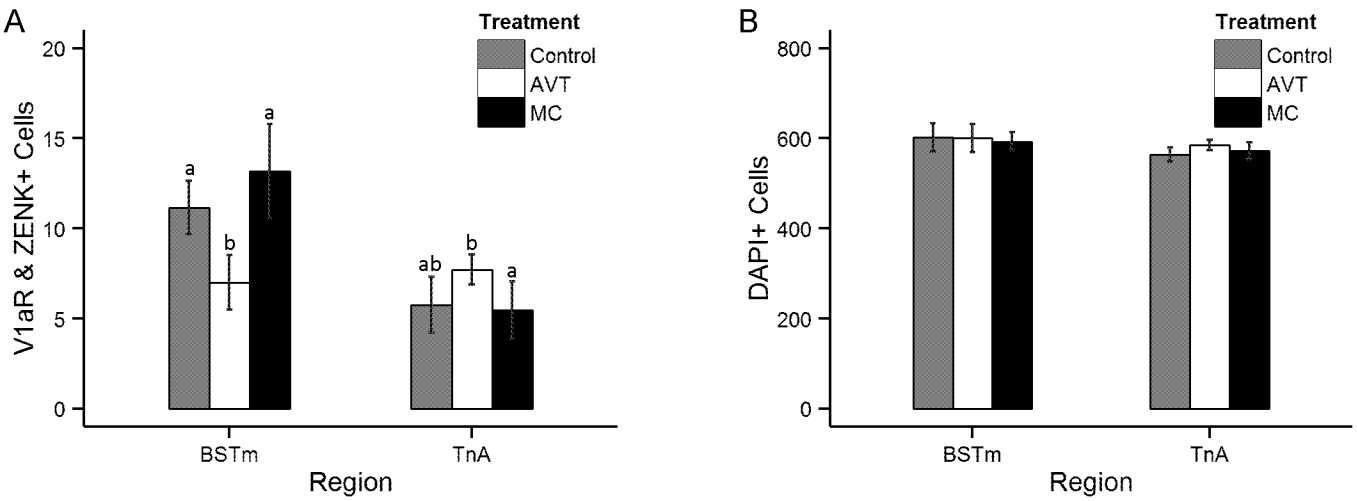

Figure 17: Number of cells expressing both V1aR and ZENK in the BSTm and TnA

A) Mean ± SE number of cells expressing both V1aR and ZENK in the bed nucleus of the stria terminalis (BSTm) and the nucleus taeniae (TnA). B) Mean ± SE DAPI-stained nuclei in the BSTm and the TnA. Subjects received intracranial injections on post-hatch days 2-8 of either arginine vasotocin (AVT), Manning Compound (MC, a V1aR antagonist), or vehicle control of saline (Control). Letters indicate groups that are significantly different from each other.

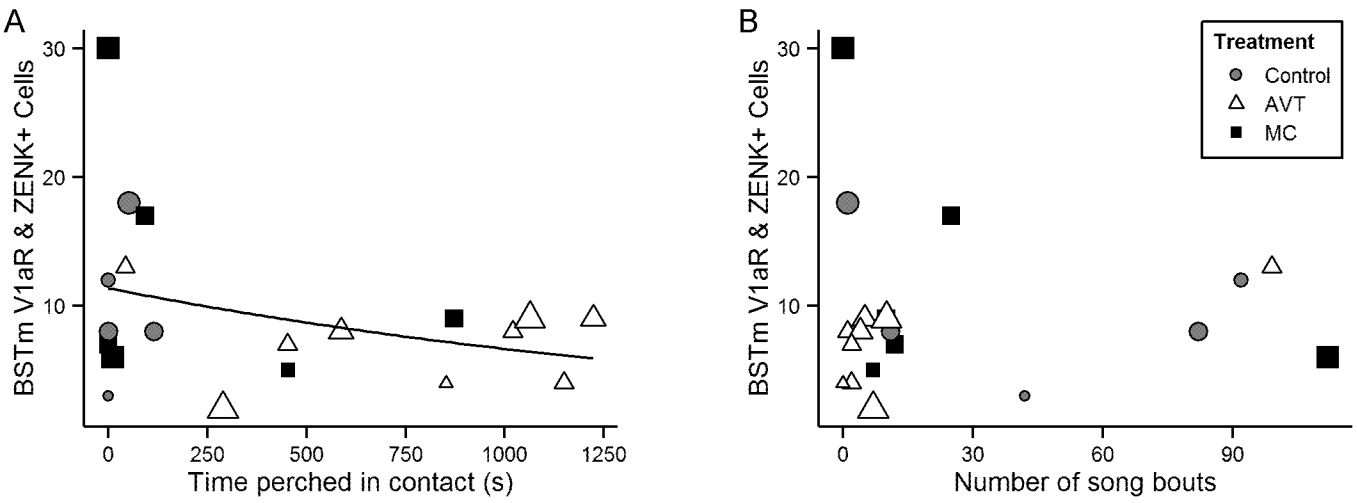

Figure 18: Number of V1aR+ZENK cells in the BSTm in relation to clumping and singing

Scatterplot of the number of cells in the bed nucleus of the stria terminalis (BSTm) expressing both V1aR and ZENK and A) time (s) spent perched in contact (clumping) with the female partner, with a line depicting a significant Poisson general linear model fit, and B) the number of song bouts during the reunion period following a one-hour separation. Subjects received intracranial injections on post-hatch days 2-8 of either arginine vasotocin (AVT), Manning Compound (MC, a V1aR antagonist), or vehicle control of saline (Control). Control subjects are depicted with gray circles, AVT with white triangles, and MC as black squares. The size of the points is scaled to the number of counting frames per subject, from the largest (n = 4) to the smallest (n = 1).

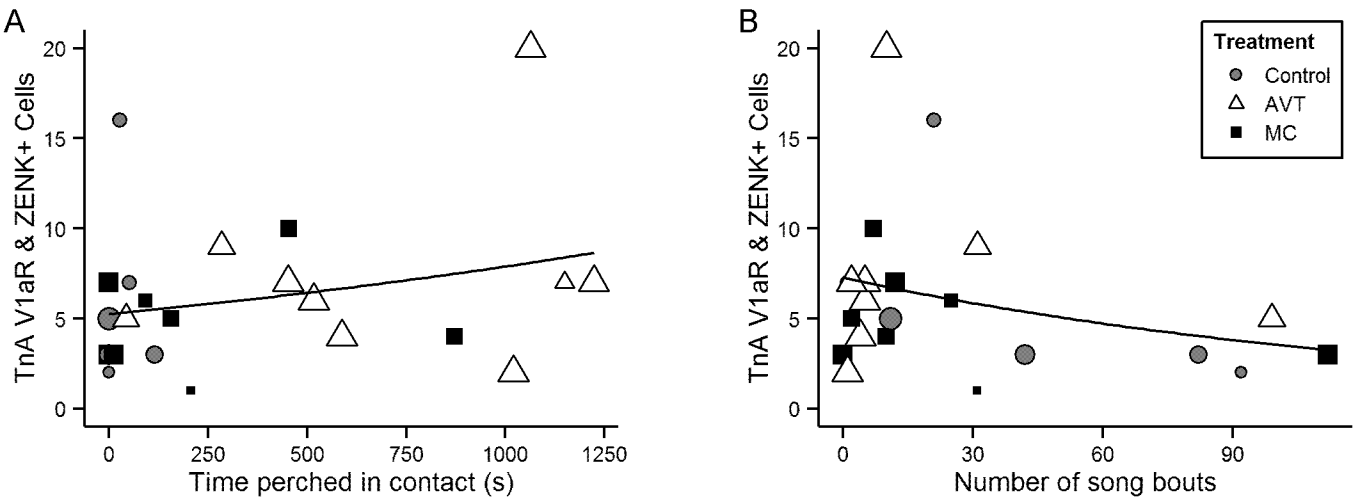

Figure 19: Number of V1aR+ZENK cells in the TnA in relation to clumping and singing

Scatterplot of the number of cells in the nucleus taeniae (TnA) expressing both V1aR and ZENK and A) time (s) spent perched in contact with the female partner and B) the number of song bouts during the reunion period following a one-hour separation. The lines depict significant Poisson general linear model fits. Subjects received intracranial injections on post-hatch days 2-8 of either arginine vasotocin (AVT), Manning Compound (MC, a V1aR antagonist), or vehicle control of saline (Control). Control subjects are depicted with gray circles, AVT with white triangles, and MC as black squares. The size of the points is scaled to the number of counting frames per subject, from the largest (n = 6) to the smallest (n = 1).

CHAPTER FIVE

Organizational effects of vasotocin and a nonapeptide receptor antagonist on song learning and courtship song in the zebra finch

Abstract: Zebra finch males learn courtship song through socially-guided feedback from adult tutors. This experiment tested the hypothesis that the nonapeptide arginine vasotocin (AVT, avian homologue of vasopressin) and nonapeptide receptors play an organizational role prior to fledging in song learning in zebra finches. In two separate experiments, hatchling zebra finch males received daily intracranial injections (post-hatch days 2-8) of either AVT, Manning Compound (MC, a potent V1a receptor antagonist and mild oxytocin receptor antagonist) or saline (vehicle control). In Study 1, we obtained song recordings from subject males between post-hatch days 90 and 97 and acoustically compared the song to the song of the adult father. Both AVT and MC males took longer to sing in a courtship context and sang fewer song bouts than Control males. Additionally, MC males had a worse match to their father's song than both AVT and Control males and there was a non-significant trend suggesting that AVT males had a better acoustic match to their father's song. In Study 2, we employed cross-fostering to replicate this experiment using a within-family design. We obtained song recordings from males on both post-hatch day 90 and day 120 to assess the learning across development. Similar to Study 1, MC males had a worse match to father's song than both AVT and Control males at both post-hatch days 90 and 120. MC males also differed from Control males in several acoustic parameters of their song itself, including amplitude, mean frequency, and a measure of periodicity. These results provide strong evidence that the nonapeptides, long thought to underlie species differences in social behaviors, may additionally play an important and thus far unexplored role in vocal learning.

Introduction:

Song learning in birds has become a model for understanding general principles underlying complex vocal learning across species, including language learning in humans. Zebra finches (*Taeniopygia guttata*), like song birds of many species, learn courtship song via socially-guided feedback from adult tutors. Over the course of development, male zebra finches learn to produce a song that closely resembles the song produced by their social father which is then used both in mate attraction and the maintenance of the pair relationship (Zann, 1996). Zebra finches learn exclusively during an early sensitive period, which lasts from approximately day 25 to 90 post-hatch (Slater et al., 1988). Early in this sensitive period, males produce 'subsong,' which progresses into more mature and structured plastic song, before ultimately "crystallizing" in the final adult song at approximately 120 days post hatch. In zebra finches, this crystallized song is highly stereotyped, and does not change during adulthood.

A great deal of research has been done on the neural mechanisms underlying song learning in birds. Both the efferent motor pathway necessary for the production of song in adult birds[1] and the anterior forebrain "loop"[2] necessary for song learning have been well characterized in song birds, particularly in the zebra finch (Brenowitz, et al., 1997; Nottebohm et al., 1990; Nottebohm et al., 1982; Nottebohm et al., 1976). Furthermore, sexual dimorphism in song nuclei size in the zebra finch brain and singing behavior appear to be organized early in development (Balthazart & Adkins-Regan, 2002). The vast majority of the research on organizational effects of hormones on song circuitry and singing behavior has focused on steroid hormones (Balthazart & Adkins-Regan,

[1] HVC → robust nucleus of the arcopallium (RA) → nucleus intercollicularis (ICo) → nucleus of the hypoglossal nerve (NXIIts) → syrinx

[2] HVC →Area X → medial nucleus of the dorsolateral thalamus (DLM) → lateral portion of the magnocellular nucleus of the anterior nidopallium (LMAN) → Area X/ RA

2002; Schlinger, 1997). However, there is convergent evidence that nonapeptides in the oxytocin family (mesotocin (MT) and arginine vasotocin (AVT) in birds; oxytocin (OT) and arginine vasopressin (AVP) in mammals) may play an important and thus far unexplored role in vocal learning and the production of socially-relevant vocalizations.

Nonapeptides have been implicated as important modulators of social behaviors across species (Goodson, 2005; O'Connell & Hofmann, 2011). Nonapeptides derive from the AVP/OT cell groups of the supraoptic (SON) and paraventricular (PVN) nuclei of the hypothalamus, as well as from smaller extrahypothalamic accessory cell groups, including the medial amygdala (meAMY), medial bed nucleus of the stria terminalis (BSTm), lateral septum (LS), olfactory bulb (OB), and suprachiasmatic nucleus (SCN) (Kelly & Goodson, 2014b). Nonapeptides produced by these cell groups modulate the activity of neurons by subsequent binding to receptors distributed throughout the brain, but importantly the distribution of these receptors and, thus, their functions are remarkably variable across species (Goodson, 2005; Kelly & Goodson, 2014b). Furthermore, the abundance of AVT/AVP elements in a number of brain regions is often sexually dimorphic (usually male greater than female), organized by sex steroids during development, and often sensitive to changes in seasonal changes in gonadal state (De Vries & Al- Shamma, 1990; De Vries & Buijs, 1983; De Vries & Panzica, 2006; Goodson & Bass, 2001; Goodson & Thompson, 2010; Kabelik et al., 2010; Kelly & Goodson, 2014a; Kimura et al., 1999). In general, AVT/AVP is more strongly associated with male behavior (see Goodson & Bass, 2001).

Across vertebrate species, alterations in reproductively-relevant vocal behavior are one of the most common and pronounced effects of AVT/AVP (Goodson & Bass, 2001). AVT/AVP has been demonstrated to affect vocalization latency and duration, as well as acoustic features of the vocalization itself in several vertebrate species. Furthermore, AVT/AVP-ir cells, fibers, and

receptors are consistently found in vocally-active brain regions (Goodson & Bass, 2000b, 2001). In plainfin midshipman fish (*Porichthys notatus*), a species in which territorial males use a long-duration rhythmic vocal "hum" to attract females, AVT administered to both a basal forebrain region (POA-AH) and the paralemniscal midbrain tegmentum was found to modulate the electrically-evoked rhythmic vocal motor output (Goodson & Bass, 2000a, 2000b). In male frogs, which use vocal advertisement calls in mate attraction and male-male spacing, both AVT and its receptors are found in every brain area implicated in vocal behavior (Boyd, 2013). Injections of AVT modulate advertisement calling frequency and call latency in all anuran amphibian species thus far investigated (Boyd, 1994; Burmeister et al., 2001; Chu et al, 1998; Kime et al., 2007; Klomberg & Marler, 2000; Marler, et al., 1995; Penna et al., 1992; Propper & Dixon, 1997; Semsar et al., 1998; Ten Eyck, 2005; Tito et al., 1999; Trainor et al., 2003). Furthermore, injection of AVT directly into the laryngeal motor nucleus stimulated calling and altered several acoustic parameters of the call (Boyd, 1994).

There is also compelling evidence that AVT is involved in vocal behaviors in adult birds. AVT injected both peripherally and centrally has significant effects on sexual and vocal behavior in Japanese quail (Castagna, et al., 1998). In male canaries, injections of an AVT analog (dGVTA, which acts as an antagonist in mammals) was found to increase singing rate only during early fall, when canaries are annually adding new syllables to their song repertoire (Voorhuis, et al., 1991). Subcutaneous infusion of AVT was found to inhibit singing and courtship in male zebra finches, which was reversed by testosterone administration (Harding & Rowe, 2003). Acute infusion of AVT into the brain was found to rapidly increase vocalizations and song in female white-crowned sparrows (*Zonotrichia leucophrys*), which normally sing only occasionally and primarily in territorial or aggressive contexts (Maney et al., 1997). Complex agonistic dawn song in male field

sparrows (*Spizella pusilla*) was significantly facilitated by infusion of AVT into the lateral septum (Goodson, 1998). In male zebra finches, the number of AVT-ir neurons and AVT-Fos co-expression in the BSTm are highly correlated with directed singing by zebra finch males to a female stimulus (Goodson et al., 2009). Collectively, these results suggest that the peripheral AVT may inhibit singing via actions on the hypothalamic-pituitary-adrenal (HPA) and hypothalamic-pituitary-gonadal (HPG) axes, but central AVT appears to more consistently facilitate singing.

In general, AVT/AVP is thought to affect social behaviors, including vocalization, by modulating multiple brain regions involved in either motivation, sensory-motor processing, or both (Goodson & Bass, 2001; Rose & Moore, 2002; Syal & Finlay, 2011). With respect to vocal learning, there is extensive evidence that song learning in zebra finches is a social process, with both motivational and sensorimotor components. In zebra finches, there is a significant overlap between sensory (memorizing) and sensory-motor (practicing) learning phases (Slater et al., 1988). Furthermore, zebra finches do not develop species-typical adult song if reared in isolation (Williams et al., 1993). Zebra finches also appear to learn better if they are given a live tutor, rather than a recording (Derégnaucourt et al., 2013; Eales, 1989). However, they can learn from a recording but only if the recorded song is played contingently based on their own song or actions (Adret, 1993). The salience of live adult tutor song is based on a wide-range of multi-modal cues including physical proximity of the tutor (Mann & Slater, 1995), aggression directed towards the fledglings (Clayton, 1987; Jones & Slater, 1996), the tutor's mating status and partner quality (Eales, 1987; Mann & Slater, 1994), visual cues (Mann & Slater, 1995; Mann et al., 1991), and auditory information, such as song similarity between the father and subsequent song tutors (Clayton, 1987). Juvenile males preferentially learn to sing from their fathers, even when other potential tutors are available. However, they have been found to copy another adult male who

provides them with more parental care (Williams, 1990). Finally, there is now increasing evidence that parents can influence juvenile learning through non-vocal proximal cues, such as wing strokes and fluff-ups (Menyhart et al., in prep).

Despite the evidence that AVT is involved in sexually dimorphic and socially-relevant behaviors across species, including vocalizations and bird song, the effects of AVT on vocal learning remain unknown. In this experiment, we test the hypothesis that the nonapeptide arginine vasotocin (AVT, avian homologue of vasopressin) and nonapeptide receptors play an organizational role prior to fledging in song learning in zebra finch males. Organizational effects of a hormone are defined as occurring early in development, when they establish the neural and physiological substrate for future behavior (Phoenix et al., 1959). Organizational effects are thought to occur during a critical window or sensitive period in development and exert permanent and long-lasting effects for the life of the individual. For example, steroid hormones have been demonstrated to partially organize the neural song system in zebra finches (Balthazart & Adkins-Regan, 2002). Estradiol treatment of female zebra finches during the first week post-hatch masculinizes the soma sizes of neurons in RA and leads to singing behavior if these females are then treated with testosterone as adults (Adkins-Regan et al., 1994; Gurney, 1982; Gurney & Konishi, 1980; Konishi & Akutagawa, 1988). Thus, we hypothesized that a similarly profound effect on song learning would result from exogenous administration of AVT or a nonapeptide receptor antagonist.

In two separate experiments, we manipulated the nonapeptide system of zebra finch chicks on days 2-8 post-hatching via daily intracranial (IC) injections of either AVT, Manning Compound [MC, a potent V1a receptor (V1aR) and mild OT receptor antagonist] or saline (vehicle control) and assessed the effect on song learning, most importantly the acoustic match to the social father.

Previously, we demonstrated that these IC injections of AVT increased affiliative interest in parents in juvenile males, which was sustained throughout development (Baran et al., in review). In contrast, MC males do not show an affiliative interest in parents at any point in development and also do not show the normal increase in affiliative interest in females as they mature. Given these effects on affiliative behavior, we predicted that AVT injected birds would show a better acoustic match to their social father's song in adulthood than Controls, whereas MC males would show a worse match. Additionally, given the correlation between AVT-ir neurons in the BSTm and directed singing (Goodson et al. 2009), we also hypothesized that manipulations of the AVT system would also affect the motivation to court females. Specifically, we predicted that MC would increase song latencies in courtship contexts and decrease total song number.

Methods:

Study 1 Breeding Conditions

The same set of male subjects used in Chapters 3 & 4 was used in Study 1. In this study, seventy-two unpaired adult males and females (hereafter "parents") were assigned to one of six breeding aviaries (1.2 x 0.9 x 0.6 m) and allowed to pair and breed. Offspring hatched within 40 days became the experimental subjects used in the study. Chicks of each sex were randomly assigned to a treatment group on day two, following genetic sexing. Starting on Day 2 post-hatching through Day 8, subjects received daily 2μL intracranial (IC) injections of either 1) AVT (10ng); 2) Manning Compound (MC), a potent V1a and mild OT receptor antagonist (50ng); or 3) 0.9% isotonic saline vehicle control (Castagna et al., 1998; Goodson et al., 2004; Manning et al., 1989). Both AVT and Manning Compound act at multiple receptor subtypes in the zebra finch brain, including the VT4 (V1aR), VT3 (OT-like), and V2 receptors (Kruszynski et al., 1980; Leung

et al., 2009; Manning et al., 2012). IC injections were performed using a sterile stainless steel insulin syringe (Beckman Dickman, U-100 BD Ultra-Fine Short Lo-Dose™ Insulin Syringes, 31 Gauge, 0.5mL volume, 8mm needle length), similar to Bender & Veney (2008).

Chicks within the same clutch were randomly assigned to different treatment groups, such that treatment was unrelated to hatching order. One Control male was found dead on post-hatch day 38 and was subsequently excluded from the experiment. The number of males that completed treatment and survived until adulthood are as follows: AVT (N = 11); Manning Compound (N = 11); Control (N = 7). After approximately 40 days of age (39.8 ± 5.4 days), subjects were removed from their natal aviary and housed in same-sex aviaries in a separate room from the parents. The only interactions that subjects with their parents after we moved them in to same sex housing was during the weekly four-way affiliative preference tests which took place from day 30 to day 86 (see Chapter 3).

Study 2 Breeding Conditions

We used cross-fostering on the second day post-hatch to create a within-family design. The genetic sex of the subject birds was determined on the day of hatching and chicks were then cross fostered on day 2 post-hatch to create families with three male subjects (one per treatment group) and one non-subject female sibling. The experiment was conducted using three temporally separate family cohorts (n = 7 families), each with a total clutch size of four.

In order to generate research subjects, ten each of male and female non-pair-bonded zebra finches (hereafter "parents") were placed into a large breeding aviary (1.2 x 0.9 x 0.6 m). Parent pairs and nest box occupancy were determined based upon the display of pair maintenance behaviors, including clumping, allopreening, and the occupancy of a nest box together. Parent

pairs to produce the offspring subjects were chosen based on breeding success, assessed via number of eggs hatched and number of chicks that survived during the pair's first breeding attempt. To create each cohort, two successful pairs were removed from the pairing aviary and placed in a separate flight aviary to allow for better observation and control. Additional pairs were kept in a separate breeding aviary and served as extra breeding birds to supplement experimental nests with nestlings. The birds were kept on a 14/10h light/dark cycle and were provided with seed, cuttle bone, water, and grit *ad libitum* throughout the study, with supplemental hard-boiled eggs weekly during the egg laying period. Additionally, each aviary was equipped with a nest box and nest-building material (coconut fiber), allowing the parent pairs to construct nests and breed.

Nest boxes were checked daily in the morning to record the number of eggs and chicks. On the day of hatching, subjects were genotyped to determine the genetic sex using DNA extracted from feather follicle tissue (similar to Baran et al., in review). All male offspring which hatched within forty days of pair-aviary assignment of the adults were used as experimental subjects, to be raised by foster parents until day 60 post-hatch. In addition, one female offspring was placed in each experimental nest to serve as a female sibling in order to create a more natural developmental setting (average clutch size is 4-5, Zann, 1996). Any additional females were cross-fostered into nests in a non-experimental aviary to be raised for future studies. All of the experimental subjects were cross-fostered and chicks were marked with non-toxic markers for individual identification. Chicks received colored leg bands for identification prior to fledging, at approximately 14 days old.

The experiment was conducted using three temporally separate cohorts (n = 7 families), with each cohort containing equal numbers of subjects from each experimental condition. Each of the three cohorts received the same experimental procedures but were temporally staggered, such that

the final subject population consisted of 21 males. One subject was excluded from the study because of incorrect genetic sexing. An additional two subjects died during the course of the experiment. One AVT subject male was also excluded from the analyses because his female parent and all of his siblings died before day 35 post-hatch, so his song learning was predicted to be abnormal. The final number of subjects at the end the experiment totaled 17, consisting of AVT (n=6), Manning Compound (n=5), and saline control (n=6) subjects. All procedures were developed with veterinary supervision and approved by Cornell University's Institutional Animal Care and Use Committee.

Study 1 Song Recordings

After reaching sexual maturity, subjects were randomly assigned an unmanipulated, sexually-naive, and unpaired female pair partner (hereafter 'partner', regardless of pair status). In order to obtain high-quality recordings of the male subjects' songs, all introductions between the subjects and their pair partner were performed on Day 90 in a room with no other birds, in a small aviary (57 x 32 x 42 cm) enclosed by sound attenuating foam. The subject was first placed in the cage, followed by the partner. Behavior (song latency, number of bouts of directed and undirected song) was scored for the 15 minutes following introduction. The pair was sometimes left in the cage for 20-45 minutes if a male subject did not sing during the first 15 minutes. After the introductions, the pair was immediately moved into an aviary in a colony room. The subjects were housed with the partner for a total of seven days. The pairing aviaries (57 x 32 x 42 cm or 61 x 36 x 43 cm) were arranged in a large bank of aviaries, such that they were visually, but not acoustically, isolated from other pairs in the room. High-quality songs were recorded using a similar method from both social fathers as well as other adult males in the breeding aviaries for comparisons to juvenile

songs.

As a result of profound effects of treatment on singing, a large number of males did not sing (AVT: *N* = 10/11; MC: *N* = 9/11; and Control: *N* = 2/7) during this introduction. Thus, we attempted to record songs from these males in the colony room over the course of the following week. Recordings were obtained either by an observer in the colony room using a highly-directional cardioid microphone (Sennheiser ME66) or from recordings of reunion with the partner following a 1hr separation (see Chapter 4). These song recordings were processed to remove extraneous noise prior to analysis. In the final analysis, we obtained high-quality song recordings from *N* = 4 AVT males, *N* = 8 MC males and *N* = 6 Control males, which is only a subset of all males in the experiment.

In addition, for all males in the experiment (AVT: N = 11; MC: N = 11; and Control: N= 7), we recorded the latency to the first song bout during the first introduction to the female partner. We also recorded both the latency to sing and the number of song bouts in the reunion after 1hr separation from the female partner, with whom they had been housed in a small pair aviary for seven days.

Study 2 Song Recordings

Songs were recorded from subjects between days 50 and 120 post-hatch. To obtain song recordings at each time point, juvenile males from each family were removed from their home aviaries and individually isolated in sound attenuation chambers overnight. The chambers were kept in a separate room and were constructed from coolers (0.94 x 0.38 x 0.38 m) internally lined with sound attenuating foam. Individual transport cages (0.46 x 0.23 x 0.25 m) along with the recording microphone were placed in the chambers. Keeping the juveniles in the room overnight

allowed them to become accustomed to the chamber and social isolation increased subsequent motivation to sing. Each chamber was equipped with overhead white LED lights, and the birds were kept on a 12/12h light/dark cycle and were provided with seed, cuttle bone, and water *ad libitum* while isolated in the chambers. The next day, a female was introduced to the cage with the male in order to elicit singing. The female was kept in the cage with the juvenile males for the entire duration of the recording session. Songs were recorded immediately following lights on. Song was recorded every three days from day 50 to 60, every ten days from day 60 until day 90, and on day 120 for 1 hour each day. The female used to elicit song was the same on day 120 as on day 90 for each male subject, to control for differing motivation to sing to different females. In addition, male subjects were kept in the sound attenuation chambers for as long as necessary to record multiple song bouts, even when that required several hours of recording. Song was recorded using a cardioid microphone (Sennheiser ME66).

Song Recording Analysis

In Study 1, one motif was cropped at random from subjects' recordings. In Study 2, ten motifs were cropped out at random from juvenile songs recorded at day 90 post-hatch and at day 120 post-hatch. Motifs with background noise, female calls, or cage noise were excluded from the sample. Also, the first motif of a song bout was not included because the first motif tends to deviate from subsequent ones (Hessler & Doupe, 1999). Introductory notes were identified and excluded from analysis. A total of four core motifs were cropped out at random from the songs recorded from the focal juvenile's father and the best core motif was chosen based on the least amount of background noise or cage noise.

AVT has been shown to affect acoustic features of vocalizations in other vertebrate species

(Boyd, 2013; Goodson & Bass, 2001), so we first wanted to test whether the treatment impacted the spectral features of the song which have good articulatory correlates. For the high-quality subject songs recorded in Study 2, we used Sound Analysis Pro (SAP) to analyze each subject's song for the following acoustic features: duration (s), amplitude (dB), pitch, mean frequency (kHz), peak frequency (kHz), Goodness of Pitch, Wiener Entropy, amplitude modulation, and frequency modulation.

As a measure of song learning success, the recordings were used to analyze juvenile song match to paternal song using Sound Analysis Pro (SAP). Sound Analysis Pro measures song similarity between juvenile and paternal song by splitting up the songs into syllables, defined as discrete sound units bounded by silent intervals. For each tutor-juvenile pair of songs, SAP calculates the probability that the goodness of match between the songs would have occurred by chance (Tchernichovski & Mitra, 2002). Our analysis focused primarily on the scores of song similarity, accuracy, and sequential match percentage. Percent song similarity is defined as the percentage of tutor sounds included in the juvenile's crystallized song. Tutors-pupil pairs typically have similarity scores between 65 and 95, whereas random pairs typically have scores ranging between 20 and 45 (Tchernichovski et al., 2000). Song accuracy is the average local similarity per millisecond across the crystallized song. Sequential match is calculated by comparing song tempo and rhythm between the tutor song and the juvenile's crystallized song (Tchernichovski et al., 2000). In SAP, the similarity, accuracy, and sequential match were determined using the Explore & Score feature segmentation tool (entropy: -9.5; FFT data window: 9.27 ms; contour threshold: 10; frequency range: 11025 Hz; advance window: 1.00 ms). The motifs of each juvenile's song were compared to the respective father motif, using the same father motif for juvenile day 90 song and day 120 song analyses.

Statistical Analyses

All statistical analyses were performed with R software (R Development Core Team 2007). In order to test for the effects of IC injections on whether or not males sang when introduced to a female, we used a chi-square test. To test the effect of IC injections on song latency data (which were not normally distributed), we used a non-parametric Kruskal-Wallis, followed by a pair-wise Wilcoxon test to perform planned comparisons between the different treatment groups. In order to test for the effect of IC injections on the number of song bouts during the reunion period, we used a generalized linear model with a Poisson link function, followed by Tukey's posthoc tests with a non-adjusted p-value to perform planned comparisons using *glht* in the multcomp package.

We used linear mixed models (LMM) to test the effect of the treatment on the acoustic features of the song and measures of similarity to the social father. We used the *lmer* function of the lme4 package (Bates et al., 2014) which allowed us to define multiple distinct random factors. In these models, Treatment (and song recording date in Study 2) were specified as fixed factors. The interaction effect considered was Treatment*Day (in Study 2 only). Random factors were individual ID (18 levels in Study 1 and 17 levels in Study 2), nested within Family ID (13 levels in Study 1 and 6 levels in Study 2). To perform model comparisons for the LMM models, we used likelihood ratio tests to compare the full model to a reduced null model with only the factor of interest removed using the *anova* function to perform a chi-square test. To test the significance of each fixed effect within a model, we used the Kenward-Roger approximation to get approximate degrees of freedom and the t-distribution to get p-values (Kenward-Roger in the pbkrtest package) and these results are presented in tables. In addition, we performed post hoc tests on the interaction terms using the *testInteractions* function in the phia package.

Results:

Song Latencies and Numbers (Study 1 only)

There was a significant effect of treatment on whether or not the male sang during the first 15 minutes of introduction to a female conspecific ($X^2(2) = 9.1$, $p = 0.01$). Five out of the seven Control males sang during the first 15 minutes of introduction to an aviary containing a female, but very few of the individuals who were treated with either AVT or MC sang at all during this introduction (AVT: N = 1/11; MC: N= 2/11). Furthermore, there was a significant effect of treatment on latency to sing (Kruskal-Wallis test: $X^2(2) = 9.26$, $p = 0.01$), such that both AVT and MC males had significantly longer song latencies than Controls (AVT-Control: $p = 0.005$, MC-Control: $p = 0.05$; AVT-MC: $p = 0.5$) (Figure 20a).

After one week of being housed with the female partner, we assessed behavior during a 25 minute reunion with the partner following a one-hour separation. There was not a significant effect of treatment on whether subject males sang at all during this 25 minute reunion ($X^2(2) = 0.68$, $p = 0.7$). However, every single Control male sang within 10 seconds of their female partner being released into the aviary, but there were significantly longer latencies in the other two groups (Kruskal-Wallis test: $X^2(2) = 7.1$, $p = 0.03$) (Figure 20b). AVT and MC males again had significantly longer song latencies than Controls (AVT-Control: $p = 0.02$, MC-Control: $p = 0.02$; AVT-MC: $p = 0.9$). Indeed, there was a significant effect of treatment on whether males sang within 10 seconds of being reunited with their female partner ($X^2(2) = 7.3$, $p = 0.03$).

There was also a highly significant effect of treatment on the number of songs sang during the reunion (Poisson GLM: $X^2(2) = 80.1$, $p < 0.0001$) (Figure 21). In general, Control males would sing some directed songs, but would also sing a large number of undirected songs. In this case, each treatment group was significantly different from each other (Control-AVT: $Z = -8.7$, $p <$

0.0001; Control-MC: Z = -3.2, p = 0.003; and AVT-MC: Z = 5.7, p < 0.0001). In contrast, AVT subjects tended to sing a small number of directed songs when reunited with the female and then would spend significantly more time perched in contact with their partner (see Chapter 4).

Acoustic Features Analysis

We tested whether treatment as well as day (day 90 versus 120) affected the acoustic features of the subjects' songs in Study 2 only, controlling for Individual ID nested within Family ID as a random variable. Results are summarized in Table 5.

Duration is a measure of the length of the core motif (not including introductory notes). There was a statistically significant interaction between treatment and day in song duration controlling for Individual ID nested within Family ($X^2(2) = 8.3$, $p = 0.02$). The overall song length decreased in the Control group between day 90 and day 120 (t = -3.2, p = 0.005), but it actually increased in the AVT group (t = 2.2, p = 0.04). AVT and MC significantly differed from each other in slope ($X^2(1) = 7.4$, $p = 0.02$) (the slope difference between Control and AVT was nearly significant).

Amplitude is a measure of loudness. There was a highly significant interaction between treatment and day in song amplitude (dB) controlling for Individual ID nested within Family ($X^2(2) = 19.9$, $p < 0.0001$) (Figure 22a). All groups became louder between day 90 and 120 (t = 10.1, p < 0.0001). AVT birds were louder on day 90 (t = 3.0, p = 0.01), but decreased less in loudness between day 90 and 120 than both MC and Control males (Control-AVT: $X^2(1) = 14.4$, $p = 0.0003$; AVT-MC: $X^2(1) = 16.2$, $p = 0.0002$).

Pitch is used to describe the perceived tone of sounds. Quantitatively, pitch estimates are measures of the period of oscillation. There was a statistically significant main effect of treatment on pitch ($X^2(2) = 7.8$, $p = 0.02$). Pitch appeared to increase slightly between days 90 and 120 (t =

2.1, $p = 0.05$). MC males had a lower pitch compared to Controls, though this effect did not quite reach significance ($t = -2.0$, $p = 0.07$).

Mean frequency is a pitch measure, which assesses the center of the distribution of power across frequencies. There was a significant interaction between treatment and day in mean frequency, controlling for Individual ID nested within Family ($X^2(2) = 8.0$, $p = 0.02$) (Figure 22b). MC males had a lower mean frequency compared to Controls ($t = -2.8$, $p = 0.009$). Additionally, both AVT and MC males, but not Controls, increased in mean frequency between day 90 and 120 (AVT: $t = 2.7$, $p = 0.01$; MC: $t = 2.2$, $p = 0.04$).

The results for peak frequency are very similar to the results for mean frequency. Peak frequency is simply the frequency of maximum power. There was a significant interaction between treatment and day in peak frequency, controlling for Individual ID nested within Family ($X^2(2) = 8.7$, $p = 0.01$). MC males had a lower peak frequency compared to Controls ($t = -2.7$, $p = 0.01$). Both AVT and MC males, but not Controls, increased in peak frequency between day 90 and 120 (AVT: $t = 2.9$, p = 0.007); MC: $t = 2.0$, $p = 0.05$).

There was a nearly significant effect of treatment on goodness of pitch, an estimate of harmonic pitch periodicity ($X^2(2) = 5.7$, $p = 0.06$) (Figure 22c). In general, the goodness of pitch actually increased between day 90 and 120 ($t = 5.4$, $p = 0.0002$). However, MC birds had a higher goodness of pitch than Control males ($t = 2.6$, $p = 0.02$). Since "goodness" of pitch is a measure of how periodic the sound is, it only captures the extent to which there are harmonic stacks in the song. Both noisy sounds and pure tones give low values for goodness. Noisy sounds, however, also give high entropy. Therefore, considering both Wiener entropy and goodness of pitch can be helpful.

Wiener entropy is a measure of the width and uniformity of the power spectrum. White noise is typically broadband with sound energy smeared rather smoothly within the noise range, whereas

animal sounds are less uniform in their frequency structure. Wiener entropy is measured on a logarithmic scale, ranging from 0 to minus infinity (white noise: $\log_1$=0; pure tone: $\log_0$=minus infinity). There was a statistically significant interaction between treatment and day in entropy, controlling for Individual ID nested within Family ($X^2(2) = 11.1$, $p = 0.004$) (Figure 22d). Wiener entropy slightly but significantly increased in the Control group between day 90 and 120 ($t = 2.5$, $p = 0.02$), but it decreased in both the AVT and MC males (AVT: $t = -2.4$, $p = 0.03$; MC: $t = -3.2$, $p = 0.004$).

Frequency modulation is the angular component of squared time and frequency derivatives, i.e. the extent to which the song includes sweeping pitch changes. There was a highly significant interaction between treatment and day in frequency modulation, controlling for Individual ID nested within Family ID ($X^2(2) = 26.4$, $p < 0.0001$). Control males appeared to increase in their frequency modulation between day 90 and 120 ($t = 2.9$, $p = 0.01$), whereas both AVT and MC males decreased their frequency modulation (AVT: $t = -2.9$, $p = 0.01$; MC: $t = -5.2$, $p = 0.0002$). Furthermore, MC males decreased their frequency modulation more than AVT males ($X^2(1) = 5.7$, $p = 0.02$).

Amplitude modulation captures changes in the amplitude envelope of sounds. There was a significant interaction between treatment and day in amplitude modulation, controlling for Individual ID nested within Family ($X^2(2) = 9.0$, $p = 0.01$). Control males slightly increased their amplitude modulation between day 90 and 120 ($t = 3.1$, $p = 0.004$), whereas AVT males decreased the amplitude modulation between day 90 and 120 ($t = -3.0$, $p = 0.005$).

Thus, the primary main effects of treatment on the acoustic features of the males' songs were on amplitude, measures of pitch, goodness of pitch, and entropy.

Study 1 Similarity Measures

There was a significant effect of treatment on the similarity score ($X^2(2) = 10.9$, $p = 0.004$) (Figure 23a). MC males were found to have a significantly lower similarity score than both Control males ($X^2(1) = 4.7$, $p = 0.03$) and AVT males ($X^2(1) = 15.1$, $p = 0.0001$). Additionally, there was a nearly significant trend for AVT males to have higher similarity scores than Control males ($X^2(1) = 3.3$, $p = 0.07$).

Similar results were observed for the accuracy score, a more fine-grained measure of local similarity. There was a significant effect of treatment on the accuracy score ($X^2(2) = 14.3$, $p = 0.0008$) (Figure 23b). MC males were found to have a significantly lower accuracy score than both Control males ($X^2(1) = 10.9$, $p = 0.001$) and AVT males ($X^2(1) = 22.9$, $p < 0.0001$). Again, there was a nearly significant trend for AVT males to have higher accuracy scores than Control males ($X^2(1) = 2.8$, $p = 0.09$). There was no effect of treatment on the measure of sequential match ($X^2(2) = 0.8$, $p = 0.7$).

Study 2 Similarity Measures

There was again a significant effect of treatment on the similarity score in this second experiment both at Day 90 ($X^2(2) = 8.5$, $p = 0.01$; Figure 24a) and at Day 120 ($X^2(2) = 9.7$, $p = 0.008$, Figure 24b). In both cases, MC males had a lower similarity score than both Control (Day 90: $X^2(1) = 8.6$, $p = 0.007$; Day 120: $X^2(1) = 7.5$, $p = 0.01$) and AVT males (Day 90: $X^2(1) = 10.3$, $p = 0.004$); Day 120: $X^2(1) = 12.5$, $p = 0.001$). There was no evidence that AVT males had a higher similarity score than Controls, though this is largely the result of a single family that represents an extreme outlier from the trend (see below). There was no effect of treatment on either accuracy score (Day 90: $X^2(2) = 1.8$, $p = 0.4$; Day 120: $X^2(2) = 0.2$, $p = 0.9$) or sequential match (Day 90:

$X^2(2) = 0.4$, $p = 0.8$); Day 120: $X^2(2) = 1.9$, $p = 0.4$).

Both studies combined

Similarity scores from day 90 songs of all individual males in both Study 1 and Study 2 are shown in Figure 25. There was no statistically significant difference between the studies for the similarity scores at Day 90, so we combined both studies to increase our power to test the effect of treatment on Similarity score and controlled for study. In this case, once again there was a highly significant effect of treatment on similarity score ($X^2(2) = 16.5$, $p = 0.0003$). Once again, MC males were found to have a significantly lower accuracy score than both Control males ($X^2(1) = 11.9$, $p = 0.001$) and AVT males ($X^2(1) = 19.1$, $p < 0.0001$). AVT males still did not appear to have a higher similarity score than Controls, though a close inspection of the data suggests that there is a single family in Study 2 in which both the Control and AVT males represent extreme outliers inconsistent with their group trends. In this family, the Control male had the highest similarity score of all Control males and the AVT male had the lowest similarity score of all AVT males (46). In fact, this AVT males is more than 5 standard deviations below the AVT group mean and his Control sibling is more than 3 standard deviations above the Control group mean. Even the MC male in this family is more than 2 SD's below the MC group mean.

We do not yet have a full understanding of why this family contains such extreme outliers. However, a preliminary look at videos of the aviary filmed during the period after the chicks had fledged suggests that the other male parent in the aviary was particularly aggressive towards all other birds in the aviary, including the chicks in this family. Thus, if we perform the same analysis removing all subjects in this aviary (the offspring of the aggressive male were already excluded because two of them died), then the significance of the overall model becomes even greater ($X^2(2)$

= 23.1, $p < 0.0001$) and each of the differences between treatment groups becomes highly significant (Control-AVT: $X^2(1) = 8.5$, $p = 0.003$; Control-MC: $X^2(1) = 11.2$, p = 0.002; AVT-MC: $X^2(1) = 37.5$, $p < 0.0001$) (Figure 25b).

Discussion:

These results provide the first evidence that the nonapeptide AVT and the V1aR have an effect on vocal learning in any species. Additionally, although AVT has been demonstrated to have acute effects on vocalizations across vertebrate taxa, these results provide the first evidence for an organizational effect of AVT on the quantity and acoustic characteristics of vocalizations. Both AVT and MC males took longer to sing in a courtship context and sang less overall compared to Control males. MC males also sang a song that was lower in mean and peak frequencies, higher in measures of periodicity, and louder on day 120 than Control males. Early life manipulations of the AVT system also affected the extent to which males' songs matched the songs of their social fathers—MC males' songs had reduced similarity, whereas AVT males' songs had higher similarity scores compared to Controls. These results are all the more remarkable given that these injections occurred long before song learning is thought to occur.

Candidate Sites of Action

There are several plausible mechanisms by which changes to the AVT system might affect vocalizations. Receptors for AVT, including V1aR and OTR, are widespread throughout the zebra finch brain, including regions that are known to be involved in auditory processing, motor production, motivation, and structures known to contribute to song learning (Leung et al., 2011, 2009)

There is evidence that both AVT and V1aR appear to be heavily involved in sensorimotor processing in the vocal domain. Several structures in the auditory forebrain, including the caudomedial mesopallium (CMM) and the caudomedial nidopallium (NCM), express high densities of V1aR mRNA in the zebra finch (Leung et al., 2011). In addition, two nuclei involved in the motor pathway of song production contain high densities of receptors for AVT. There is AVT-ir in the intercollicular nuclues (ICo, a region implicated in vocal control) in several species, as well as consistent evidence of AVT binding in the region (Kiss, et al., 1987; Leung et al., 2009; Panzica et al., 1999; Voorhuis & De Kloet, 1992). Additionally, the key motor nucleus, nXIIts, which innervates the syrinx and is considered to be part of the song system, contains high levels of mRNA for all three subtypes of VT receptor mRNA (Leung et al., 2011). The expression of AVT receptors in these motor regions help to account for the effects of AVT on acoustic features of the song.

However, given the significant effects of AVT and MC on similarity scores, it is perhaps surprising that there is in fact only limited evidence that the anterior forebrain pathway (AFP) involved in song learning is directly modulated by AVT. In the bird species where this has been investigated, there is in fact minimal expression of receptors for nonapeptides in the AFP and, when they are present, notable variability in expression levels across species. There is no evidence for AVT binding in either medial dorsolateral thalamus (DLM) and Area X (Leung et al., 2011, 2009). There is no evidence of AVT binding in LMAN in zebra finches, though there was found to be high production of both VT1 and V1aR mRNA in this region (Leung et al., 2011, 2009). Both white-throated sparrows and zebra finches do exhibit evidence of VT3 (OT-like) receptor binding in HVC (Leung et al., 2011, 2009)., though this is unlikely to be the primary site of action for the present effects given that more significant effects were found using the V1aR antagonist.

Finally, AVT binding within RA ranges from none to moderate and appears to be somewhat variable even within species (Leung et al., 2009; Voorhuis & De Kloet, 1992). However, the identification of the receptors underlying this binding remains elusive, with evidence that either VT2 or V1a (VT4) receptor subtypes may be present in RA in several species (Leung et al., 2011, 2009; Voorhuis & De Kloet, 1992).

There is evidence in support of somewhat indirect modulation of song learning circuitry by AVT in songbirds. There is a high density of AVT-ir fibers and V1aR expressed in the ventral tegmental area (VTA) of zebra finches, which plays a critical role in on-line song learning (Leung et al., 2011, 2009; Voorhuis & De Kloet, 1992). Dopamine neurons in VTA send a large projection to Area X within the striatum and this projection is hypothesized to play a critical role in reinforcement learning as a juvenile zebra finch attempts to match his own vocalizations to the memory trace of his tutor's song (Fee & Goldberg, 2011; Gale & Perkel, 2010). Thus, AVT may affect a juvenile male's ability to re-create his father's song by modulating a critical component of this basal ganglia-thalamocortical loop.

Furthermore, across several species of songbirds, AVT-ir fibers are found in the capsular region surrounding RA (RA is thought to be homologous to laryngeal motor cortex) and/or the arcopallium directly ventral and medial to RA (Kimura et al., 1999; Kiss et al., 1987; Pfenning et al., 2014; Plumari et al., 2004; Voorhuis & De Kloet, 1992; Voorhuis et al., 1991). This AVT-immunoreactivity is testosterone dependent in several songbird species, including the zebra finch (Kimura et al., 1999; Plumari et al., 2004; Voorhuis et al., 1988). This suggests that the AVT projections to this region most likely originate from extra-hypothalamic sources like the BSTm or LS, which are more often sexually dimorphic and steroid dependent. The presence of both vasotocin and tyrosine hydroxylase (TH) immunoreactive fibers in the ventral arcopallium

(Appeltants et al. 2001) has led to the suggestion that these dopaminergic and vasotocinergic fibers may originate from the same neurons in the medial preoptic area (mPOA) (Riters & Alger, 2004), though this remains to be tested.

There is still a great deal of debate about the microcircuitry and function of this region of arcopallium just ventral to RA, which is colloquially known as the "RA cup." This area receives input from primary auditory cortical field L1 and HVC-shelf in the nidopallium, as well as the mPOA (Kelley & Nottebohm, 1979; Mello et al., 1998; Vates et al., 1996). In one study, a distinct population of cells in this region, identified in one study as the ventral intermediate arcopallium (AIV), appears to project to the dopaminergic midbrain (VTA and SNc), which then projects to Area X (Gale et al., 2008; Mandelblat-Cerf et al., 2014). Neurons in AIV exhibit very rapid responses to auditory feedback during singing and lesions to this region result in disruptions to song learning (Mandelblat-Cerf et al., 2014). In addition, there appears to be expression of VT2, VT3/OT-like and VT4/V1aR receptors in the arcopallium surrounding RA (Leung et al., 2011, 2009; Voorhuis et al., 1988).

Thus, it is clear that AVT can modulate regions that participate in both the sensory processing and production of song. Furthermore, there are several candidate regions whereby AVT may heavily modulate song learning circuitry. However, much future work is needed to determine if this can account for the present effects of early manipulations of the AVT system on learned vocalizations.

Motivation and Social Context

However, there is a key component of the song learning process that is often underappreciated: motivation. Why is it that a juvenile zebra finch is motivated to pay attention to the song of a tutor

or other non-vocal cues from conspecifics at the time song learning is occurring? In all species that exhibit vocal learning, social environment is a critical component of this process. The nonapeptide systems, particularly AVT, appear to provide an important mechanism to be able to modulate the motivation to produce song in particular social contexts. For example, the connections between the amygdala (including AVT cell groups in the medial amygdala and BSTm) and projection from VTA to the nucleus accumbens (NAcc) form a large part of the mesolimbic dopamine pathway. This pathway modulates, among other things, behavioral responses to stimuli that activate feelings of reward or motivation. Thus, it is thought that the nonapeptides provide a critical linkage between the motivation and social circuits, thus providing a system that can represent and attach motivational value to social conspecifics (O'Connell & Hofmann, 2011; Syal & Finlay, 2011).

Zebra finches are highly gregarious and experience a high degree of overlap in the memorization and acquisition phases of song learning (Roper & Zann, 2006; Slater et al., 1988), allowing the opportunity for social feedback to influence learning. Despite the extensive investigation of both song learning in zebra finches and the role of nonapeptides in a wide range of social behaviors, the role that the nonapeptides may play in motivating social behavior has rarely been investigated in a developmental context. Previous research has demonstrated that manipulations of the AVT system early in life resulted in significant alterations to affiliation with and attachment to the parents (see Chapter 3). We found that MC males exhibit less affiliative interest in their parents throughout development and the present results demonstrate that they also have impaired song learning. These findings provide a mechanism that could account for the differences between treatment groups in song learning. Indeed, the song produced by MC males actually resembles the song produced by males raised without a tutor. Zebra finches raised in isolation develop a song with abnormal properties, including unusual note structure and decreased

stereotypy (Price, 1979; Williams et al., 1993). Untutored songs also often include repeated notes, resembling the structure of the trills of canaries (Williams, 2004). Similar to untutored song, MC males had higher within-subject variability in their song. Furthermore, several of the MC males exhibited abnormal repeated syllables at the beginning of their core motif, which partially accounts for their low similarity scores.

Thus, the decreased similarity to the father and increase in unusual note structure observed in the MC males may be a function of inattention to behavioral feedback from conspecifics, rather than an absence of a song model altogether. This is also consistent with the interpretation that MC does not result in motor impairment per se, but exerts its effects on song learning by changing MC males' attention to socially-relevant cues during song learning.

In contrast, the song produced by AVT males in general is a better acoustic match to father's song than the song sung by Control males. This was a nearly significant trend in the first experiment (based on only 4 AVT male songs) and was highly significant across both studies when an outlier family was excluded from analyses. If this trend is real, this is also consistent with the findings from Baran et al. (2015) that AVT males exhibit enhanced motivation to affiliate with the parents as juveniles. Indeed, this mechanism could act both through closer attention to the song of the tutor, and also through improved attention to non-vocal cues from females. Non-singing female listeners are also known to affect song learning (Jones & Slater, 1993). Males raised with deaf adult females sing more frequently and develop more atypical songs than those raised with hearing females (Williams, 2004), and blindfolded males raised with a tutor develop more accurate song when also raised with a female sibling than when raised without one (Adret, 2003). These cases of enhanced learning in the presence of conspecifics may be the result of heightened arousal or attention in social contexts (ten Cate, 1991), or the result of attendance to song-elicited conspecific

behaviors (Vyas et al., 2009). A closer investigation of the learning process will shed light on the proximal mechanisms underlying this improved learning in AVT males.

It is also in the context of motivation that we can interpret the results demonstrating that both AVT and MC affect song latencies and numbers. Although both AVT and MC males show decreased singing when first introduced to a female conspecific, important differences are revealed in the reunions with their partner following a brief separation. AVT males tended to exhibit a small number of directed songs and then spent a significantly increased time perched in contact with their female partner (see Chapter 4). Control males sang very quickly when being reunited with their partner and sang at much higher rates, but perched in contact with their partner significantly less than AVT males. In contrast, MC males exhibited lower rates of both perching in contact and singing. This again suggests that early life manipulations of the AVT system may alter the extent to which different behaviors are exhibited in similar social contexts. This can be interpreted as AVT, MC, and Control males all employing different pair maintenance strategies. In this case, song is just one possible behavior in the repertoire for re-establishing the pair bond.

Thus, one interpretation of these results is that changes to the AVT system in early life affect the particular context in which song is used. The activity of AVT neurons in the BSTm has been demonstrated to be associated with higher rates of directed singing (Goodson et al., 2009). This suggests that the production of AVT by neurons in the extended medial amygdala may be important for modulating the specific social contexts in which song is or is not exhibited and raises the possibility that these early life injections have perhaps permanently altered processing of social context by BSTm neurons and, thus, their modulation of singing behavior through their widespread actions on vocally-active brain regions. However, this hypothesis remains to be tested.

Conclusion:

These results provide the first demonstration that the nonapeptides, long thought to underlie species differences in social behaviors, may additionally play an important and thus far completely unexplored role in vocal learning. Despite the lack of strong evidence for direct vasotocineric modulation of the classical song system, these results suggest that a critical component of the song learning circuit remains poorly understood. Furthermore, they provide strong support for the case that social factors may be critically important in understanding the development of vocalizations in naturalistic settings. There is an unfortunate tendency among researchers in the neurobiology of bird song to view song learning as merely a motor challenge, in which birds are working to match their vocal production to the template that they have stored in memory. This research has to a large extent ignored the important role that motivation and social circuits play in the learning process. This view is bolstered by increasing behavioral work suggesting that contingent social feedback from both male tutors and non-vocal cues from females is important in vocal learning.

Syal & Finlay (2011) proposed that one of the major innovations necessary for the evolution and development of vocal learning is the linkage between the neural representation of central caregivers (such as the zebra finch tutor) and the motivational systems. Although these results provide evidence consistent with this hypothesis, much future work is needed to further elucidate the exact mechanisms of these effects. However, there are a large number of brain regions where AVT is predicted to modulate singing in the zebra finch. Indeed, as the avian model of song learning becomes an increasingly important window into vocal learning in humans, these findings suggest that understanding the modulation of vocal circuits by motivational and social circuits will be critical.

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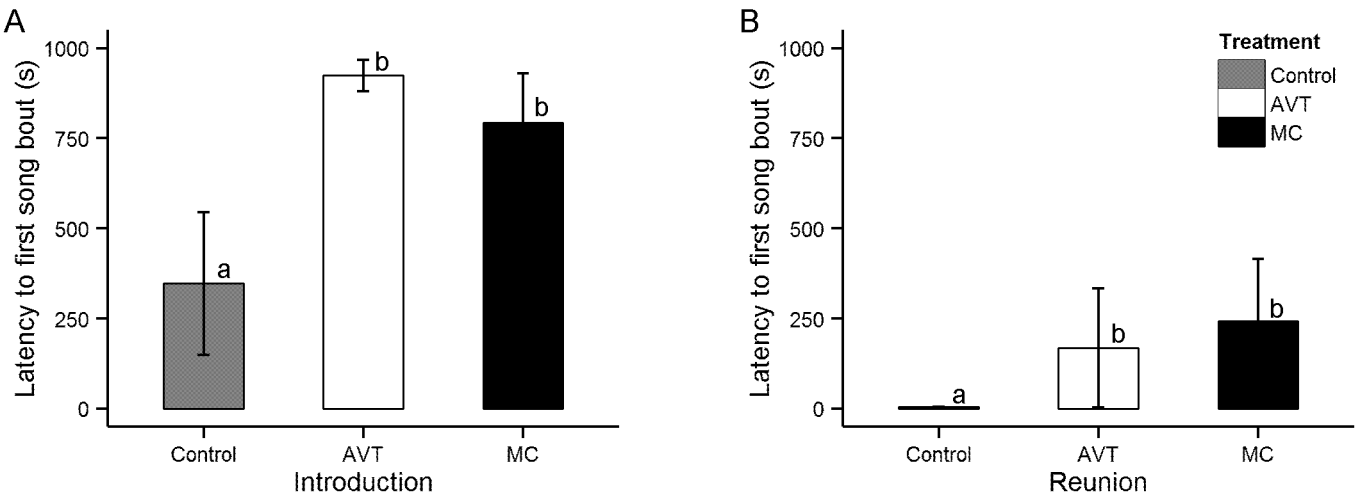

Figure 20: Latency to sing when introduced and later reunited with a female partner

Mean ± SE of the latency in seconds until the males' first song bout during A) introduction to a novel female conspecific ("partner") and B) reunion with the female partner following a one-hour separation after being housed with that female for one week. Letters indicate groups that are significantly different from each other.

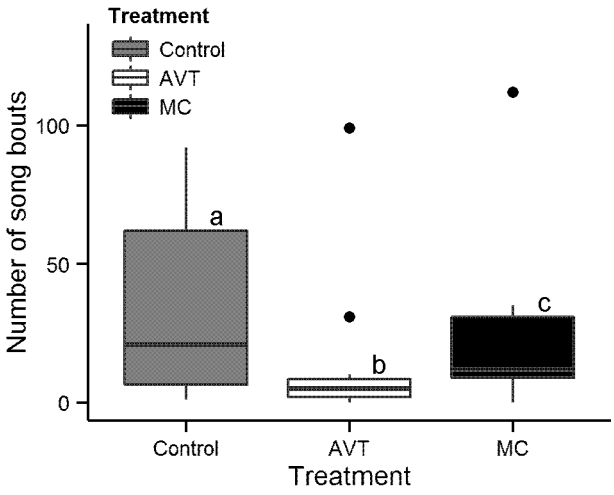

Figure 21: Number of song bouts during reunion with the female partner

Box-and-whisker plot of the number of song bouts during the first 25 minutes following reunion with the female partner after a one-hour separation. Data beyond the end of the whiskers are outliers and plotted as points. Letters indicate groups that are significantly different from each other.

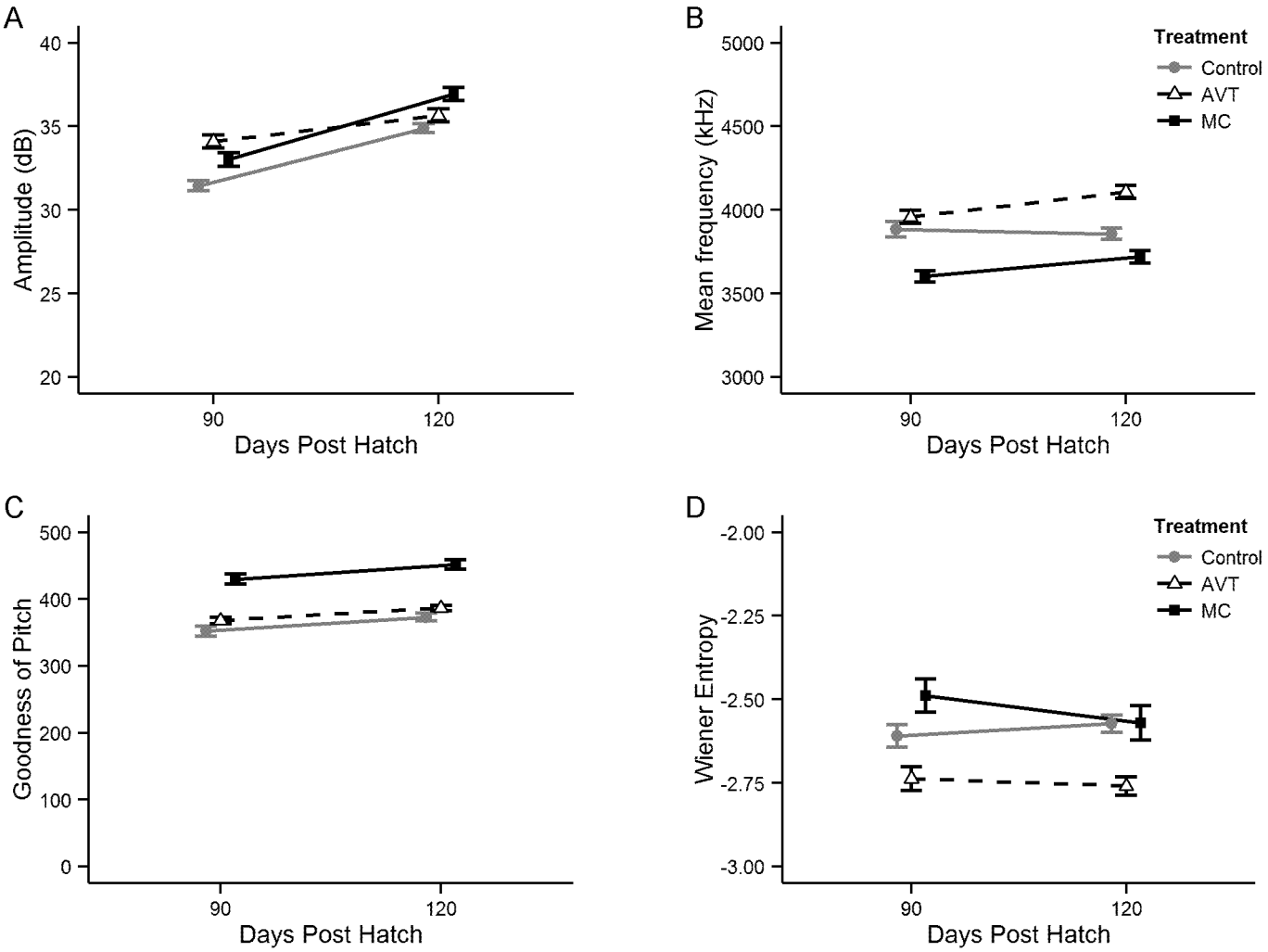

Figure 22: Acoustic features of song recorded on day 90 and day 120 post-hatching

Mean ± SE of A) the amplitude (dB) of the song motif, B) the mean frequency (kHz), C) Goodness of pitch, and D) Wiener entropy as measured using Sound Analysis Pro (SAP). Control subjects are depicted with circles and a solid gray line, AVT with triangles and dashed black line, and MC as squares and solid black line.

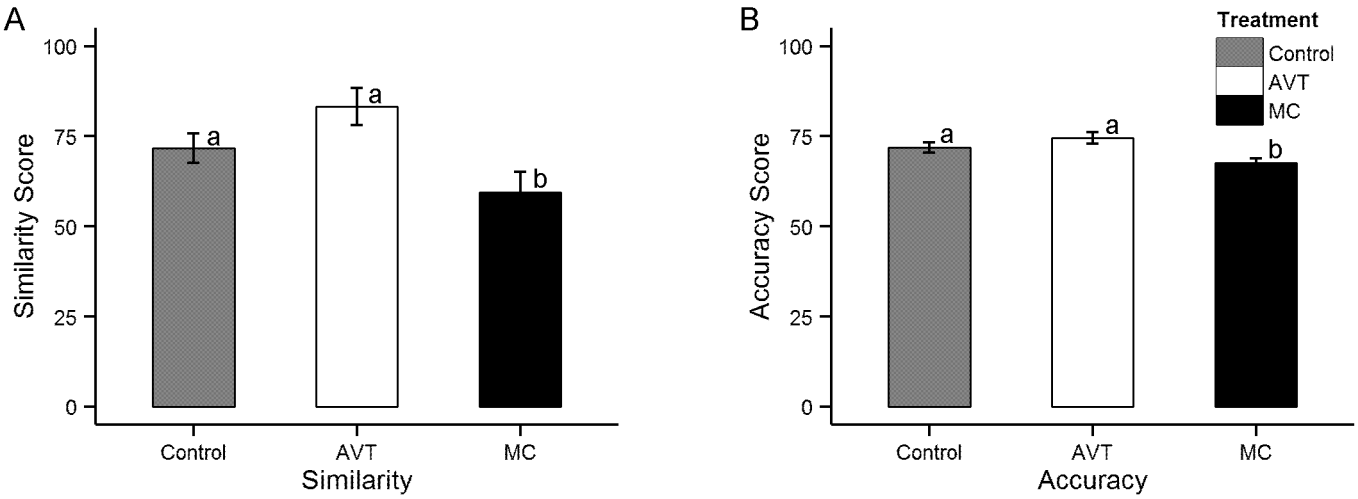

Figure 23: Study 1 acoustic match to father's song

Mean ± SE of A) the similarity score and B) the accuracy score between juvenile's and his social father's song as calculated using Sound Analysis Pro (SAP). The similarity score is defined as the percentage of tutor sounds included in the juvenile's crystallized song. Song accuracy is the average local similarity per millisecond across the crystallized song. Letters indicate groups that are significantly different from each other.

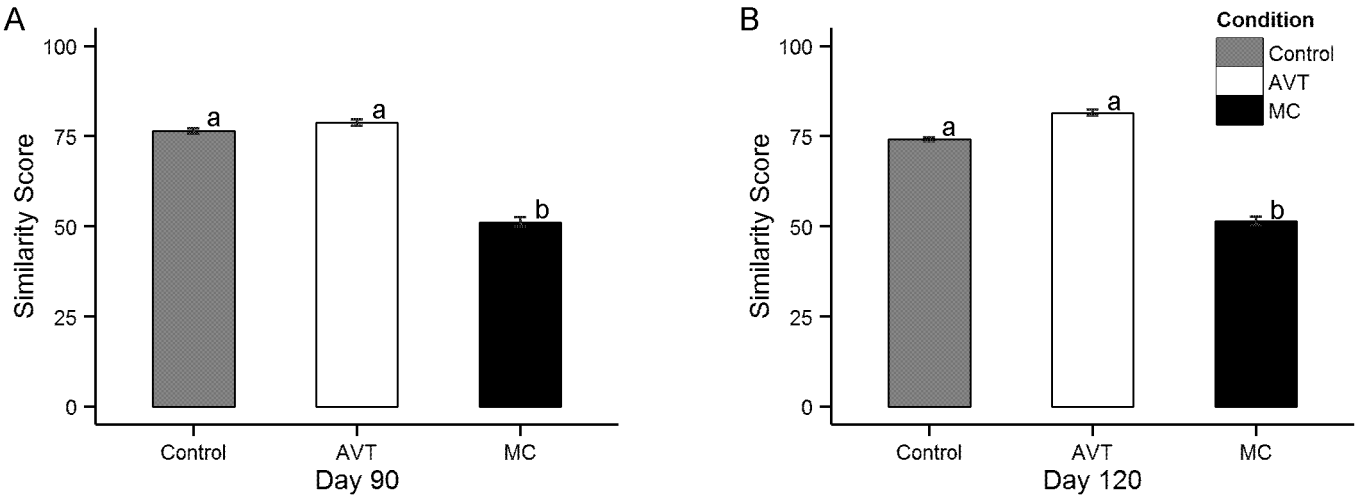

Figure 24: Study 2 acoustic match to father's song

Mean ± SE of the similarity score between juvenile's and his social father's song as calculated using Sound Analysis Pro (SAP) for songs recorded on A) day 90 and B) day 120 post-hatching. The similarity score is defined as the percentage of tutor sounds included in the juvenile's crystallized song. Letters indicate groups that are significantly different from each other.

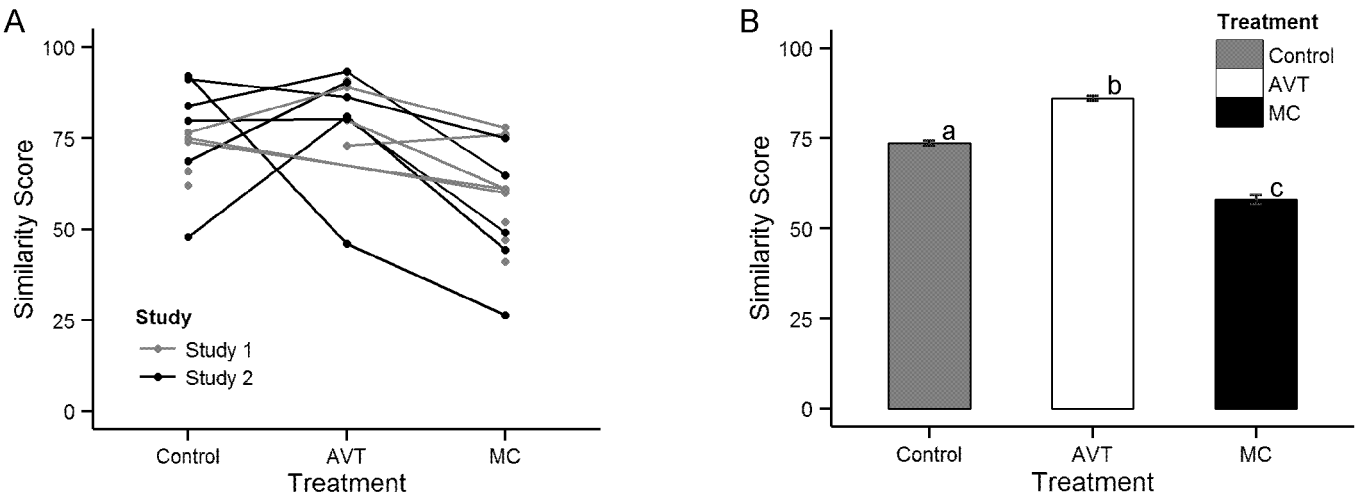

Figure 25: Similarity scores combining both Study 1 and Study 2

Panel A depicts the individual similarity score from each male's day 90 song in both Study 1 and Study 2. The similarity score is defined as the percentage of tutor sounds included in the juvenile's crystallized song. Lines connect siblings within the same family. The similarity score for subjects in Study 1 is from a single song recording, whereas the mean similarity score is shown for subjects in Study 2. Subjects from Study1 are depicted in gray and subjects in Study 2 are depicted in black. Panel B depicts the mean ± SE of the similarity of subjects from both studies, excluding subjects in the outlier family. Letters indicate groups that are significantly different from each other.

	Duration(s)				*Amplitude (dB)*				*Pitch*			
Predictors	Estimate	SE	*t*	*p*	Estimate	SE	*t*	*p*	Estimate	SE	*t*	*p*
Intercept	737.249	88.109	8.367	**0.000**	31.169	0.987	31.585	**0.000**	1186.953	93.270	12.726	**0.000**
DPH (120)	-52.210	16.470	-3.170	**0.005**	3.715	0.366	10.142	**0.000**	27.173	12.717	2.137	**0.048**
Treatment (AVT)	-104.705	124.546	-0.841	0.410	3.187	1.079	2.954	**0.011**	124.776	94.786	1.316	0.207
Treatment (MC)	90.143	130.548	0.690	0.497	1.801	1.141	1.578	0.139	-197.378	100.791	-1.958	0.068
Treatment (AVT)* DPH(120)	51.562	23.296	2.213	**0.038**	-1.968	0.518	-3.798	**0.002**				
Treatment (MC)*DPH(120)	-12.670	23.676	-0.535	0.598	0.152	0.527	0.288	0.778				

	Mean Frequency (kHz)				*Peak Frequency (kHz)*				*Goodness of Pitch*			
	Estimate	SE	*t*	*p*	Estimate	SE	*t*	*p*	Estimate	SE	*t*	*p*
Intercept	3861.651	63.142	61.158	**0.00E+00**	3839.487	70.819	54.216	**0.00E+00**	352.743	23.606	14.943	**1.50E-08**
DPH (120)	-5.069	38.757	-0.131	0.897	-11.629	43.921	-0.265	0.793	19.905	3.700	5.380	**0.000**
Treatment (AVT)	93.177	88.839	1.049	0.303	105.937	99.628	1.063	0.297	22.449	25.784	0.871	0.403
Treatment (MC)	-258.672	92.597	-2.794	**0.009**	-278.558	103.830	-2.683	**0.012**	71.680	27.366	2.619	**0.024**
Treatment (AVT)* DPH(120)	145.944	54.795	2.663	**0.013**	179.942	62.094	2.898	**0.007**				
Treatment (MC)*DPH(120)	121.286	55.746	2.176	**0.038**	127.605	63.173	2.020	0.053				

	Wiener Entropy				*Frequency Modulation*				*Amplitude Modulation*			
	Estimate	SE	*t*	*p*	Estimate	SE	*t*	*p*	Estimate	SE	*t*	*p*
Intercept	-2.667	0.134	-19.883	**2.15E-14**	39.109	1.529	25.571	**1.67E-11**	-0.010	0.001	-8.882	**1.07E-10**
DPH (120)	0.094	0.038	2.459	**0.023**	1.075	0.368	2.923	**0.013**	0.002	0.001	3.077	**0.004**
Treatment (AVT)	-0.060	0.156	-0.385	0.704	2.095	1.439	1.455	0.172	-0.001	0.002	-0.862	0.394
Treatment (MC)	0.163	0.165	0.989	0.335	1.652	1.530	1.079	0.302	-0.002	0.002	-1.036	0.307
Treatment (AVT)* DPH(120)	-0.130	0.054	-2.408	**0.026**	-1.502	0.520	-2.888	**0.014**	-0.003	0.001	-3.012	**0.005**
Treatment (MC)*DPH(120)	-0.177	0.055	-3.227	**0.004**	-2.769	0.529	-5.237	**2.39E-04**	-0.001	0.001	-1.278	0.209

Table 5: Linear mixed model (LMM) results for acoustic features of song

Summary of the linear mixed models with the individual acoustic features (Duration, Amplitude, Pitch, Mean Frequency, Peak Frequency, Goodness of Pitch, Wiener Entropy, Frequency Modulation, and Amplitude Modulation) as dependent variables. The fixed effects are Treatment, Day Post-Hatch (DPH), and the interactions. Individual ID nested within Family ID was included as a random effect. The LMM models were selected based on model comparisons using likelihood ratio tests. To test the significance of each parameter within the models, we used the Kenward-Roger approximation to get approximate degrees of freedom and the t-distribution (SE = standard error, bold numbers indicate significance, * refers to an interaction term).

CHAPTER SIX

Sensitive periods, nonapeptides, and the evolution and development of social behavior

Abstract: In this chapter, I argue that the nonapeptides, by providing a way to modulate the activity of specific neural circuits in specific social contexts, may provide an important mechanism underlying the evolution and development of diverse social phenotypes across vertebrate taxa. Although the nonapeptide system has been the focus of a great deal of research over the last several decades, the vast majority of this work has focused on adults. And yet, more than any other class of neurochemicals, the nonapeptides have been found to underlie of behavioral plasticity and social diversity, including seasonal variation, sex differences, and species divergence in behavior. To frame this argument, I synthesize comparative work on the nonapeptides in adults, as well as my own work on the organizational effects of arginine vasotocin (AVT) and the V1a receptor on social behaviors in the zebra finch, to make an argument about how the nonapeptides may actually support the development of diverse social behavior by providing a functional link between motivational and social circuitry and sensorimotor processing during development. Thus, I attempt to situate my research within the newly emerging field of the evolution and development of social behavior.

Introduction:

Both extrinsic and intrinsic experience broadly shape the functional organization of the brain. Functional maturation depends critically on input at particular points in time to ensure that nervous systems are organized such that the organism is able to survive and reproduce in maturity. The basic plan of the brain is, of course, governed by genetic expression. However, its development is influenced at every point along the way by the "environment," broadly construed (Stiles, 2008).

The development of the brain is shaped by experience that comes from outside the organism, but also by chemical signals that are generated by the organism itself (which are, of course, genetically specified). These chemical signals include chemical gradients which guide the growth of neuronal projections to their targets, or longer distance chemical messengers that modulate the activity of complex neural circuits. Importantly, chemical messengers such as hormones or neuromodulators, provide a signal to coordinate the development of multiple tissues. Thus, there are important parallels between the organizational role that hormones play at during development (intrinsic experience) and the organizational effects of experience that comes from outside of the organism (extrinsic experience).

There is however little research investigating the mechanisms that govern why organisms seem to be so exquisitely sensitive to particular kinds of sensory input at specific points in development. In particular, there is a significant gap in the literature in identifying the neural mechanisms that are involved in modulating sensitive periods in development, even for potent forms of early learning like imprinting. What actually modulates the timing, existence, and nature of these sensitive periods in development, particularly given the significant impact that early social learning has on adult phenotype?

In this chapter, I argue that the nonapeptides, which provide an evolvable mechanism for modulating the activity of whole neural circuits in specific social contexts, may provide an important and so far poorly understood role in the evolution and development of diverse social phenotypes across vertebrate taxa. Although the nonapeptide system has been the focus of a great deal of research over the last several decades, the vast majority of this work has focused on adults (Choleris et al., 2013). And yet, more than any other class of neurochemicals, the nonapeptides have been found to underlie of behavioral plasticity and social diversity, including seasonal variation, sex differences, and species divergence in behavior (reviews: De Vries & Boyle, 1998; Foran & Bass, 1999; Goodson, 2005; Goodson & Bass, 2001; Insel & Young, 2001; Keverne & Curley, 2004; Lim et al., 2004; Panzica et al., 2001).

Fortunately, there is a re-emerging interest in the effects of the nonapeptides during development (Cushing, 2013). The hypothesis that the nonapeptides may exert organizational effects on the brain by producing long term or permanent changes in neural structure was first proposed during the 1980s (arginine vasopressin: Boer, 1985; oxytocin: Noonan et al., 1989). Despite several intriguing findings which suggested that the nonapeptides were important during development, there was relatively interest in this area until recently. This resurgence was largely driven by the realization that exogenous administration of synthetic oxytocin commonly used to induce labor may have unknown side effects at a critical point in brain development (Carter et al., 2009; Kenkel et al., 2014). Furthermore, it was realized that the nonapeptides may also have potential relevance to understanding social deficit and neurodevelopmental disorders (Insel, 2010).

From both of these perspectives—the comparative approach and the developmental approach—there is enough evidence to suggest that further work into the organizational effects of the nonapeptides in the evolution and development of social phenotypes will prove fruitful. In

particular, I propose that the nonapeptides may play a critical role in actually modulating sensitive periods for social learning. To frame this argument, I will first describe the classical work on sensitive periods in development. Second, I will summarize the principles of hormonal organization of the brain, leveraging the now classic work demonstrating that steroid hormones play an organizational role in the sexual differentiation of brain and behavior. Third, I will use this framework to formulate several hypotheses about how nonapeptides may play a similar organizational role in the development of social behavior. I will support this claim by synthesizing comparative work on the nonapeptides in adults, as well as my own work on the organizational effects of arginine vasotocin (AVT) and the V1a receptor on social behaviors in the zebra finch, to make an argument about how the nonapeptides may actually support the development of diverse social behavior by providing a functional link between motivational and social circuitry and sensorimotor processing. Finally, I attempt to situate these ideas within the newly emerging field of the evolution and development (evo-devo) of social behavior. In order to understand the incredible diversity of social behavior—that is, the diversity of mating systems, parental care, aggression, cooperation, territoriality, aggressive behaviors, and vocal learning across taxa—we must first understand how the social brain develops.

Sensitive periods in development:

There is extensive evidence of early sensitive periods in development, in which an organism demonstrates a marked susceptibility or vulnerability to particular stimuli during a limited time window early in life (Bornstein, 1987). This phenomenon likely reflects a developmental phase of built-in competence for specific exchange between an organism and its environment. This is most often observed in the context of what is known as experience-expectant learning, where an

organism depends on certain types of experience in order to develop normally. By experimentally removing or altering the stimulation to which the developing organism is sensitive, we can reveal the extent to which an organism requires the species-typical intrinsic or extrinsic input for species-typical development. For example, visual deprivation early in life in a number of species can cause disorganization of the cortical columns necessary to process visual stimuli (Wiesel & Hubel, 1963). Additionally, certain cues in an organism's environment may provide information about which phenotype will be most successful given the environment the organism is likely to encounter. In this case, developing organisms "sample" their environment during sensitive periods for cues which can be used to shape their development in an adaptive direction. For example, early nutritional stress can serve to program physiological function in ways that would enhance postnatal survival under conditions of intermittent or poor nutrition (Mcmillen & Robinson, 2005).

Some of the most striking examples of sensitive periods in development occur in the social domain. One particularly potent form of an early sensitive period is imprinting. Imprinting, such as filial or sexual imprinting, is defined as a form of learning that 1) can only take place during a restricted window of time in an individual's life, 2) is irreversible, 3) involves the learning of species-specific or individual-specific characters, and 4) may occur at a time when the appropriate behavior itself is not yet performed (Immelmann, 1972). Visual imprinting phenomena have been best studied in birds (Bolhuis, 1991; Burley, 2006; Lorenz, 1937; ten Cate & Vos, 1999; Vos et al., 1993; Witte & Caspers, 2006), where the circuit underlying filial imprinting has been well characterized (Horn, 1998; Knudsen, 2004; Nakamori et al., 2013). In addition, there is work from a number of fish species demonstrating imprinting phenomena, primarily in the olfactory domain (Barnett, 1986; Gerlach et al., 2008; Gerlach & Lysiak, 2006; Hasler et al., 1978; Keller-Costa et al., in press.; Kozak et al., 2011; Mann et al., 2003; Verzijden & ten Cate, 2007). Although the

olfactory system has been well characterized in the zebra fish (Yoshihara, 2014), there is surprisingly little work investigating the neural mechanisms underlying olfactory imprinting phenomena.

There are also more subtle forms of sensitive periods in development, in which experience with caregivers early in life shapes later social relationships (Ainsworth, 1989; Bowlby, 1960; Champagne et al., 2003; Zayas et al., 2011). Across taxa, isolation from conspecifics and caregivers results in significant disruptions to social functioning later in life (Bertin & Richard-Yris, 2005; Harlow et al., 1965; Lévy et al., 2003; Rutter, 1998). Furthermore, research in rats has demonstrated that early experiences of maternal care (e.g. licking and grooming behaviors) can alter the responsiveness to stressors in these rats in adulthood, as well as their maternal behavior (Champagne et al., 2003; Liu et al., 1997; Meaney, 2001).

What appears to be common across these different kinds of sensitive periods in development is competitive exclusion (Bateson & Hinde, 1987). In other words, a particular class of sensory input from the environment is favored, excluding others. It is important to note that these early forms of social learning, particularly about the identity, features, and valence of caregivers, is an important foundation for later learning. Learning the characteristics of and maintaining the motivation to be proximal to caregivers provides developing organisms with food and protection, but also a plethora of opportunities for later social learning. In these cases, we may have a sense of what circuits are involved in the sensitive periods (as well as a number of ways to disrupt those circuits), but critically we do not know the mechanisms of how or why there is a particular kind of sensitivity in the first place. For example, the fillial imprinting work in birds has focused on forebrain regions thought to be homologous to mammalian cortex which are involved in visual processing and multimodal association (Horn, 1998; Knudsen, 2004; Nakamori et al., 2013). Of

course, multimodal association regions would be engaged in learning about sensory input in multiple modalities, but why those particular inputs at that particular time?

Principles of hormonal organization:

Hormonal mechanisms are prime candidates for the mediating sensitive periods in development. A number of hormones, including the glucocorticoids, sex steroids, and the nonapeptides, have been shown to be involved in learning and memory, both directly and indirectly (Martinez, Jr. et al., 2006). Hormones can act directly on the cellular processes of neurons, but they can also affect more general systems such as arousal or energy function. Across several species that show olfactory imprinting, for example, hormonal changes are often coincident with the period in which learning is facilitated (Hudson, 1993).

Because they coordinate the expression of suites of physiological, morphological and behavioral traits, hormones are well-suited to play this role in sensitive periods. Hormone acts as signals, which can influence multiple tissues called targets because these tissues contain receptors and necessary co-factors for the hormone to act (Ketterson et al., 2009). A hormonal signal can have major effects on physiological and developmental processes on targets across a wide spatial and temporal distance. This is of great potential benefit, allowing the organism to coordinate target tissues of different types, such as whole neural circuits which can be simultaneously recruited for an important task (Adkins-Regan, 2005).

An important theme in the field of behavioral neuroendocrinology is the distinction between organizational versus activational effects of hormones (Phoenix et al., 1959). According to the organizational/activational hypothesis, hormonal events early in development are responsible for the induction of certain types of behavioral patterns by establishing the neural and physiological

substrate for future behavior (organization). In adulthood, the actions of the same hormones are also necessary for causing the expression of the behavior by activating, modulating or inhibiting the function of these existing circuits (activation). The timing of the organizational effects of hormones is usually restricted to critical periods in development in which the organism is maximally susceptible to the organizing effects of hormones and these effects are often permanent, lasting for the life of the individual. The timing of critical periods in hormonal organization is ultimately determined by both the presence of the hormone and the readiness of the tissue to respond the signal. In some cases, the onset of the hormonal signal itself can induce the production of the necessary receptors and co-factors to ensure that a given tissue is in the right state to be able respond to respond to that hormonal signal.

Most of the organizational versus activational effects of hormones have been studied in the context of steroid hormones and sexual differentiation (although other hormones like juvenile hormone, thyroid hormone and gonadotropin-releasing hormone (GnRH) have also been studied in development). Classic work in guinea pigs demonstrated that androgens administered during gestation resulted in a decrease in the female-typical lordosis copulatory posture when primed with estrogen and progesterone and instead increased the female's display of male-typical mounting behaviors when treated with testosterone (Phoenix et al., 1959). Similarly, in male Japanese quail whose eggs were injected with either testosterone or estradiol prior to day 12 of the 17 day incubation period are demasculinized and exhibit fewer male-typical copulatory behaviors, whereas female quail treated with anti-estrogen exhibit male-typical copulatory behaviors (Adkins, 1976; Adkins-Regan, 1987; Adkins-Regan et al., 1982). Additionally, numerous studies have demonstrated that gonadal steroids can create some of the sex differences in singing behavior and the neural song system (Balthazart & Adkins-Regan, 2002).

However, these organizational hormone effects have not been extensively studied in relation to learning, despite extensive evidence that aspects of sexual behavior is learned (Woodson, 2002). Thus, there remains a gap in our understanding the role that hormones play in learning and memory processes generally and the organizational role that they may play in influencing the outcome of development in the context of social experiences.

Overview of the nonapeptides:

Over the last several decades, a great deal of work has been devoted to understanding the vasotocin family of neuropeptides, which includes arginine vasotocin (AVT, found in non-mammals and likely the ancestral peptide) and its mammalian homolog arginine vasopressin (AVP); and the oxytocin-like peptides (isotocin (IT), found in fish, mesotocin (MT), found in lung fish and non-eutherian tetrapods, including birds; and oxytocin (OT), found in mammals) (Acher et al., 1970, 1972)[1]. The nonapeptides, as their name would suggest, are small nine-amino acid peptides. In theory, we know more about the properties of the nonapeptide-producing neurons in the supraoptic and paraventricular nuclei of the hypothalamus than any other neurons in the brain. This is because the large size of the aptly named magnocellular neurons of the hypothalamus makes them relatively easy to manipulate experimentally. For many decades, research efforts were focused on the critical role that the nonapeptides play in regulating multiple physiological functions in the body, including water balance and the stress response. However, it was several decades before researchers discovered the important contribution of these hormones to social behaviors and cognitive functioning when acting on neurons in brain.

[1] For the purposes of this chapter, I will focus primarily on AVP/AVT, but many of the general principles of my argument are likely to apply across nonapeptides.

The nonapeptides derive from an evolutionarily ancient neuromodulator. In the earliest vertebrates, only one gene was present, but sometime after *Agnatha* (lampreys and hagfish) a gene duplication event led to the divergence of the vasotocin (AVT/AVP) and oxytocin (IT/MT/OT) lineages (Moore & Lowry, 1998). Although these two lineages differ in only a single amino acid, the nonapeptides appear to have evolved quite distinct functions.

This is because the nonapeptides co-evolved with their receptors. Nonapeptide receptors are G protein-coupled receptors, with a central core structure comprising 7 trans-membrane helices connected by three intracellular and three extracellular loops. There are typically four receptor subtypes for the nonapeptides within each species: V1a, V1b, V2, and OT (VT4, VT1, VT2, and VT3, respectively, in birds). The amino acid sequences of each receptor subtypes are more similar to each other across species (~90%) than they are to different subtypes within a single species (~45%) (Darlison & Richter, 1999). The sequencing of the vertebrate nonapeptides illustrates that the core-ligand receptor interaction sites have remained remarkably conserved, while allowing the intracellular components to vary (Cho et al., 2007). Thus, when binding to their receptors, nonapeptides can have a multitude of effects on neurons including changes to gene transcription, recruitment of intracellular calcium, neuroprotective effects, and alterations to long term potentiation mechanisms (see Neumann & van den Burg, 2013 for review). Receptors for nonapeptides are distributed throughout the brain, but importantly, the distribution of each of the receptor subtypes can vary widely by species, sex, age, and social context. Additionally, because nonapeptide receptor subtypes have high degrees of structural homology with each other, their receptor binding is relatively promiscuous, which has important consequences for the actions of nonapeptides on neural circuits.

The primary sources of nonapeptides derive from the AVP/OT cell groups of the supraoptic (SON) and paraventricular (PVN) nuclei of the hypothalamus, as well as from smaller extrahypothalamic accessory cell groups, including the medial amygdala (MeA), medial bed nucleus of the stria terminalis (BSTm), lateral septum (LS), olfactory bulb (OB), and suprachiasmatic nucleus (SCN) (Choleris et al., 2013; Laycock, 2009). The abundance of AVT/AVP elements is often sexually dimorphic (usually male greater than female), organized by sex steroids during development, and sensitive to changes in gonadal state (De Vries & Al-Shamma, 1990; De Vries & Buijs, 1983; De Vries & Panzica, 2006; Goodson & Bass, 2001; Goodson & Thompson, 2010; Kabelik et al., 2010; Kelly & Goodson, 2014; Kimura et al., 1999). In general, AVT/AVP is more strongly associated with male behavior (see Goodson & Bass, 2001).

It is helpful to think of there as being three separate and semi-independent pools of AVT/AVP in vertebrates, separable by their sites of action and, ultimately, their function: 1) physiological regulation of water balance, 2) potentiation of the hypothalamic-pituitary-adrenal (HPA) axis, and 3) central actions. These pools can be thought of as separate because there is in fact relatively little leakage between the separate compartments, although the brain and the blood tend to be somewhat coupled (Neumann et al., 1993). The blood-brain barrier is highly effective at preventing AVT/AVP released into the bloodstream from acting on neurons in the brain (though there are certainly more indirect pathways that these hormones can influence the brain).

AVT/AVP, when released by magnocellular neurons in the paraventricular nucleus (PVN) and supraoptic nucleus (SON) of the hypothalamus, is released into the posterior pituitary and from there enters general circulation. In the body, AVT/AVP plays a significant role in water balance and vasoconstriction, leading to its more common clinical name, antidiuretic hormone (ADH).

Although the function of AVT/AVP in water balance was the first function discovered, research suggests that the nonapeptides were involved in sexual and reproductive functions first, and later involved their physiological role in maintaining water balance as organisms adapted to life in fresh water (Hanoune, 2009).

AVT/AVP, when it is released from the anterior pituitary by parvocellular neurons in the paraventricular nucleus of the hypothalamus (PVN), is at the top of the hypothalamic-pituitary-adrenal (HPA) axis. The HPA axis regulates the physiological response to stressors, helping the body mobilize resources in response to challenges in its environment. AVT/AVP, along with corticotropic releasing factor (CRF) serves as a releasing hormone for adrenocorticoprin-releasing hormone (ACTH) from the anterior pituitary, which is the chemical signal that leads to the release of glucocorticoids from adrenal tissue (Buckingham, 2009). AVT/AVP is not itself the major releasing hormone for ACTH, but it plays a critical role by potentiating the biological activity of CRF (Gillies & Lowry, 1982). Parvocellular AVT/AVP neurons are highly responsive to stress (Shibata et al., 2007). Acute stress increases the production of both CRF and AVT/AVP in the PVN (Harbuz et al., 1994; Xia & Krukoff, 2003).

In addition, multiple cell groups in the brain contribute to the central pool of nonapeptides. Some of the same neurons that project into the pituitary also send projections back into the brain. In addition, there are several extra-hypothalamic accessory cell groups that produce the nonapeptides, as well including the medial amygdala (meAMY), medial bed nucleus of the stria terminalis (BSTm), lateral septum (LS), olfactory bulb (OB), and suprachiasmatic nucleus (SCN) (Choleris et al., 2013; Laycock, 2009). The variation in AVT/AVP function across species or phenotypes is likely a function both the differential involvement of the separate AVT/AVP cell groups and the modulation of the interconnected set of brain nuclei known as the social behavior

network (Goodson & Kabelik, 2009; Newman, 1999). Nonapeptides can modulate neurons in a paracrine fashion when released from the axons, dendrites, and soma of AVT/AVP cells, but they are also present at high levels in the cerebrospinal fluid, allowing them to act on cells lying along the ventricles. Each of the AVT/AVP cell groups has a different pattern of activity and neural release, which is ultimately a function of the kinds of computation neurons in that region perform (Kelly & Goodson, 2014). It is also important to note that individual populations of AVT/AVP neurons may be able to independently modulate specific nuclei via targeted projections.

Nonapeptides in development

Nonapeptides appear to play a role in species differences in many diverse social behavior domains (Campbell et al., 2009; Dewan et al., 2011; Dewan et al., 2008; Goodson et al., 2009; Insel et al., 1994; Landgraf et al., 2003; Lim et al., 2004; Oldfield et al., 2013; Parker & Lee, 2001; Reddon et al., 2015; Wang et al., 1994). However, the vast majority of this comparative work has captured only snapshots of nonapeptide function in adulthood (Cushing, 2013). Thus, a substantial missing piece of the puzzle is the role that the nonapeptide hormones are playing during development to shape social behaviors, particularly those behaviors for which plasticity, flexibility and learning are critical.

Given that the nonapeptides underlie diversity in behavioral phenotypes both within and across species, an important question is how do these systems develop? Although we have good information about the development of these systems in rodents (primarily in rats, but also a more modest amount from prairie voles and mice), there is only a limited understanding of the role that these hormones may play in organizing the developing brain, particularly in social contexts.

In rats, the neurons of the SON and PVN have formed before birth by fetal day 12-14 (gestation in 21 days in the rat) (Buijs et al., 1980). The first AVP staining is observed in the rat brain between fetal day 14 and day 18, which steadily increases to adult levels by postnatal day 30 with a dip near birth (Buijs et al., 1980; Szot & Dorsa, 1993). Indeed, between birth and postnatal day 21, there is a 22-30 fold increase in AVP production by the pituitary, suggesting that the neurons that project from the hypothalamus to the pituitary are gradually coming on-line during development. In contrast, the cell groups of the BSTm and medial amygdala seem to come online only after birth, with the MeA delayed relative to the BSTm. AVP mRNA was only observed in the BSTm on postnatal day 3 and in the MeA on day 5 in male rats and day 14 and day 35, respectively, in female rats (Szot & Dorsa, 1993). The levels of AVP, thus, reach adult levels by postnatal day 35 in the BSTm and day 60 in the medial amygdala in both sexes (Szot & Dorsa, 1993). Additionally, binding sites were found in the developing mouse and rat brain in both the amygdala and septum between postnatal day 0 and 8, as well as several brain regions where AVP receptors are not expressed in adulthood, including the hippocampus, dentate gyrus, and caudate nucleus (Hammock et al., 2013; Petracca et al., 1986).

Research in rodents provides evidence consistent with the hypothesis that nonapeptides may modulate many brain regions early in development, particularly those relevant for social behavior. In rats, the beginning of production of AVP/OT from the extrahypothalamic sources and central nonapeptide receptor expression is coincident with important milestones in early social attachment and learning in rat pups (Blass, 1987; Buijs et al., 1980; Hammock et al., 2013; Petracca et al., 1986; Szot and Dorsa, 1993).

Additionally, a limited number of experimental manipulations of nonapeptides during development in rodents provide further evidence for the organizational hypothesis. Vasopressin-

deficient Brattleboro rat pups show hyperactivity, reduced huddling and reduced proximity to other pups in the nest compared to wild-type rats (Schank, 2009). Wild-type rat pups treated with a nine-day exposure to AVP showed increased emotionality, activity levels and grooming in an open field test as juveniles, as well as smaller overall brain size (Boer et al., 1994). Acute central administration of AVP in wild-type neonatal rat pups was found to decrease the number of ultrasonic vocalizations and reduced locomotor activity in a maternal isolation test (Winslow & Insel, 1993). In juvenile male rats, both targeted infusion of AVP into the LS and intracerebroventricular infusion increased preference for investigating novel individuals, whereas a V1aR antagonist increased the preference for investigating familiar individuals (Veenema et al., 2012). In addition, V1aR blockade in the LS increased social play behavior in males and decreased it in females, but only when it tested in a familiar environment (Bredewold et al., 2014; Veenema et al., 2013). In addition, neonatal manipulation of AVT or OT in the socially-monogamous prairie vole, leads to significant changes in nonapeptide binding in several brain regions in adults and alterations to social behaviors (Bales & Carter, 2003a; Bales & Carter, 2003b; Bales et al., 2007; Stribley & Carter, 1999; Yamamoto et al., 2004). These results provide intriguing evidence that the nonapeptides may be involved in the development of social behavior or early social learning, but the data are far too scarce to demonstrate a causal connection.

Conservation and novelty:

Vertebrates exhibit a remarkable diversity of behavior and social phenotypes: mating systems, parental care, aggression, cooperation, parental care, territoriality, aggressive behaviors, and vocal learning. The constant appearance of specialized behavioral phenotypes seems to be in conflict with the highly conserved structure and scaling in the evolution of vertebrate brains. Indeed, this

conservation of the scaling in size of individual brain regions is so pronounced, that 90% of variance of the size of any brain region can be accounted for by the size of any other referent brain region (Workman et al., 2013). Thus, it would seem that we cannot fully account for the evolution of behavioral novelty in evolution simply by assuming that new structures were added or even merely enlarged. This suggests that evolution has not generated phenotypic novelty by adding novel structures, but by using existing structures in new and different ways.

Sensorimotor processing hypothesis

One way to think about the contribution of nonapeptides (or any hormonal signal) is to think about both the signal that is being communicated and the tissues or structures that are on the receiving end of that signal. In other words, what information is being communicated by AVT/AVP cell groups? And what tissue needs to be sensitive to that information?

The specific functions of the individual AVT/AVP cell groups to social behaviors are still poorly understood because manipulations of endogenous production of the nonapeptides from individual cell groups are quite rare (Kelly & Goodson, 2014). However, the cell groups that are the central sources of nonapeptides are situated within structures for which we do have some understanding about their inputs and computational properties. For example, the medial amygdala receives a large projection from the accessory olfactory system in rodents and less dense projections from the main olfactory system, insular cortex, and other portions of the amygdala (Swanson & Petrovich, 1998). The PVN receives projections from limbic structures (including the lateral septum and the medial amygdala), the circumventricular organs, and other hypothalamic regions, including the medial preoptic nucleus (Silverman et al., 1981). Because the activity of neurons within these regions are also modulated by signals from other hormonal systems,

including the hypothalamic-pituitary-adrenal (HPA) and hypothalamic-pituitary-gonadal (HPG) axes, these structures are well-placed for processing and integrating features of the social and environmental context (Morimoto et al., 1996; Newman, 1999).

What about the receiving end? The expression of receptors in number of different brain structures allows the nonapeptide cell groups to modulate the activity of whole neural circuits in a coordinated fashion. This is broadly consistent with the sensorimotor hypothesis of Rose and Moore (2002), which posits that AVT/AVP can act on sensory pathways to modulate the responsiveness of neurons to particular kinds of sensory stimuli as well as act on motor pathways to modulate behavioral output. This theory emerged from comparative work which suggests that AVT can modulate each step of sensory-motor processing in a circuit controlling a complex courtship behavior in male newts (*Taricha granulosa*) (Rose & Moore, 2002). In these newts, AVT enhances the highly-stereotyped dorsal amplectic clasping behavior, in which the male firmly embraces the female with all four limbs to induce receptivity (Moore & Zoeller, 1979). Interestingly, AVT enhances this behavior by modulating multiple components of the behavior, both in sensory processing in the visual and olfactory domains as well as motor output at the level of the spinal cord (Boyd & Moore, 1991; Lowry et al., 1997; Thompson & Moore, 2000).

A parallel story may also be true in another species-specific behavior: bird song. In this case, each step in the circuit controlling the expression of complex vocal-learning in birds appears to be partially modulated by AVT, though a sensory-motor account remains to be tested (Leung et al., 2011; Leung et al., 2009; Nottebohm et al., 1976).

Social Gating Hypothesis

Thus, variability in the expression of nonapeptide receptors across species implies that evolutionary novelty may arise when new structures or circuits are modulated in new ways starting early in development. Syal and Finlay claim that what is necessary for the evolution of novel behaviors is that the sensory and motor circuits are attached to the socio-motivational circuitry (2011). In fact, they view the reciprocally-connected network of brain nuclei known as the social behavior network (which includes the major nonapeptide cell groups), as the conserved neural structure that assembles the relevant sensory dimensions of a representation of an individual and attaches that representation to motivations and actions appropriate to their social context (Syal & Finlay, 2011). In this context, the hormonal signal produced by the nonapeptide cell groups, by acting on receptors throughout the brain, can be used to bias attention towards certain kinds of sensory stimuli or to reward the performance of certain behaviors. Consequently, it is easy to imagine how even tiny tweaks to the system, such as gene mutations that change the regulation of a receptor gene or slightly alter down-stream functions of the receptor, might have large effects on whole neural circuits. Thus, the nonapeptide system may provide a mechanism whereby evolution generates novel social behavior using an otherwise highly-conserved brain.

The effect of changes to the nonapeptide system would be expected to be even more consequential in development, particularly when coupled with salient social experiences. If indeed nonapeptides are gating social learning, then the nonapeptides may function by biasing a young organisms' attention towards the behaviors exhibited by their family or other socially-relevant conspecifics. This increased attention would ultimately provide opportunities for social learning, which could also be reinforced by socio-motivational circuits. Thus, early sensitivity to social stimuli could then be used to support future social learning, leading to a ratcheting up of learning

about the features and value of interactions with conspecifics. On the other hand, genetic mutations that reduce social approach or attention during development might reduce the probability that predictable aspects of the social environment are learned.

Of course, these kinds of effects depend critically on the kinds of input that an organism receives from its environment. It is possible to think of the organizational effects of nonapeptide circuitry independent of its social environment, but more likely, the kinds of social experiences an organism and their sensitivity to those social experiences co-evolved with each other. For example, this increased interest in affiliating with parents means that juveniles learn better from the social interactions that they have with their parents, which makes these interactions more rewarding, which leads to more opportunities for social learning.

Thus, understanding the role of neuromodulators during development provides a critical link between what have often been considered three separate phenomena: differences between species, within-species differences of the categorical kind (i.e. types), and continuous variation in individual differences. Neuromodulatory systems during development have evolved to allow organisms to plastically respond to their environment as they mature. However, these evolvable systems may also provide the raw material for evolution to act, by allowing for variability in functional outcomes in adulthood.

Organizational effects of AVT on zebra finch social behavior:

The results presented in this dissertation on the organizational role of nonapeptides on a broad suite of social behaviors in the zebra finch provide support for this view. Intracranial injections of either AVT or Manning Compound (MC, a V1aR antagonist) in hatchlings appear to have altered social interest in the parents after fledging, suggesting that the nonapeptides are serving to gate a

number of social approach behaviors in juvenile zebra finches. This change in the affiliative interest in parents has predictable functional consequences for social learning. Male zebra finches injected with MC as hatchlings both showed decreased interest in their parents during development and ultimately sang a song that was a worse acoustic match to their father's song in adulthood compared to Controls. In contrast, AVT males showed increased affiliative interest in their parents and family and appear to better match their father's song. This suggests that the nonapeptides may bias the motivation of developing zebra finches to attend to the behaviors of the father during development, which ultimately allows them to more accurately learn courtship song from their father. These findings are ultimately consistent with the Syal and Finlay's hypothesis that the nonapeptides gate complex vocal learning in song birds by altering social motivation, supporting their suggestion that the nonapeptides may play an equally critical role in language learning in humans (Syal & Finlay, 2011).

The organizational effects of AVT on pairing behaviors can also be interpreted in this context. Manipulations of AVT early in life appears to have led to remarkably divergent reproductive strategies in adult male zebra finches. AVT males showed an increased affiliative interest in females as they reached reproductive maturity similar to Control males. However, they showed less sexually-motivated courting of females compared to Controls, but instead formed highly affiliative pair bonds with their female partner. In contrast, MC males did not show the normal increase in affiliative interest in females as they reached maturity and showed only modest levels of both courtship and affiliation in their interactions with females. Taken together, these findings suggest that AVT-injected males may have had more experience attending to social cues or a stronger association between affiliative interactions and reward compared to both MC and Control males, resulting in different approaches to reproduction.

This kind of variability in reproductive strategies is not uncommon within species, but it has remarkable implications for how novel social phenotypes may evolve via relatively simple alterations to the actions of a single nonapeptide during development. The effects of these manipulations on the adult brain also provide support for this idea. AVT appears to alter early social behaviors, affecting the opportunities for social learning. However, it also has effects on the organization of the neural substrate underlying these social behaviors. The sensitivity of the extended medial amygdala to AVT as well as its activity have been permanently altered by injections of the hormone shortly after hatching—a classic organizational effect.

Conclusion:

In general, the results presented in this dissertation provide support for the idea that the actions of nonapeptides in development may play an important role in the evolution of novelty in social behavior. The field of evolutionary developmental biology (evo-devo) has long been concerned with how evolution shapes developmental processes to generate phenotypic novelty. However, the insights from evo-devo have rarely expanded into the social domain (Finlay, 2007; Hofmann, 2010; Toth & Robinson, 2007). We are just barely scratching the surface in our understanding of the diversity of neural and developmental mechanisms underlying behaviors.

Indeed, the nonapeptides are almost certainly not the only chemicals that play a role in the evolution of diverse social phenotypes. We now know of more than 100 different peptides and other signaling molecules, each of which is expressed in only a small population of neurons, and all of which signal to neurons throughout the brain via specific receptors. The endless forms of neural systems and behavior thus appear to be result of evolutionary changes to compartmentalization, sub-functionalization, and modification of neuromodulatory signaling

systems (Katz & Lillvis, 2014). However, the complex nature of diverse signaling systems suggests that they can only be fully understood by integrating research at all levels of analysis—investigating both their molecular and developmental mechanisms, as well as their adaptive significance in the life of an organism.

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