Pair bonding: what mediates its formation and maintenance?

by

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ABSTRACT OF THE DISSERTATION

Pair bonding: what mediates its formation and maintenance?

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Pair bonding is an exclusive mating relationship associating the memory of a mate with the potential successful completion of a breeding cycle. In evolutionary biology, pair bonding has been studied as a mating strategy and this biological phenomenon has been associated with survival related reproductive behaviors, however, behaviors that function in the formation of bonding and those that are established as a result of bonding are seldom distinguished, making it difficult to assess how pair bonding is represented in the brain. This thesis assesses pair bonding in ring doves, an animal that has a predictable sequence leading to a successful breeding cycle. Through a series of lesion, immunohistochemistry, and behavior studies we sought to understand the processing and execution of this fitness-critical complex behavior, by assessing the neural basis of pair bonding, understanding which behavioral events initiate the formation of the bond and how bonding affects an animal's decisions involving a mate. We found a neuro-marker for pair bonding that is more accurate than current methods of measuring bonding. Lesions to this region, the nucleus taeniae, disrupted pair bonding in doves, suggesting that the nucleus taeniae is causally linked to pair bonding. Using the neural marker for pair bonding, we found that ring doves formed a pair bond following the performance of courtship (nest coo)

behavior, rather than the completion of the entire breeding cycle as previously determined (Morris and Erickson 1971). The nest coo phase of the breeding cycle triggers the hypothalamic-pituitary response in female doves culminating in ovulation (Cheng et al 1998; Cheng and Balthazart 1982), suggesting that doves are using this stage to predict the successful completion of the breeding cycle with their mate, a concept consistent with pair bonding's role in survival behavior. We found that quality of the female, whether she is a bonded mate versus a novel female, affects the amount of courtship behavior performed by the males. Male doves that have formed a pair bond perform less amount of courtship (nest coo) behavior towards their mate than male doves that are not bonded do towards a strange female, consistent with the observation that bonded doves can skip earlier parts of the courtship routine because they have already successfully completed a breeding cycle. Paradoxically, we show that this attenuation of nest coo behavior, in response to the value of the female stimulus (bonded mate versus strange female) , diminishes the production of new neurons following lesions to the VMN, the brain region moderating reproductive behavior, thereby slowing the process of synaptogenesis and, in turn, recovery of behavior after damage to this area.

Preface:

The idea for my thesis stemmed from casual conversations with my advisor regarding what it means to be pair bonded. Although pair bonding manifests itself differently across varying pair bonded species, the ultimate goal of forming a bond is the same, to ensure the reproductive success of the individual. If this was the case, pair bonding could be more than just a conglomeration of behaviors that occurs following sensory input from the bonded mate. Ultimately my thesis led me to ask: can pair bonding be represented in the brain as its own concept and, therefore, be measured in its own right, rather than measured by the behaviors which it produces? I set out to answer this question, the product of which can be found in the sections that follow.

I could not have accomplished this undertaking without the guidance and support of my advisor, Mei Fang Cheng, who allowed me to follow my interests and offered advice and encouragement throughout my time in graduate school, in particular while I was writing my thesis.

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Introduction:

An abstract entity is often difficult to describe and even more complex to measure. Such is the case with "pair bonding". Early definitions of pair bonding characterize it as an exclusive mating relationship between two animals, disregarding occasional extra-pair copulations that might occur outside of the bond (Kleiman 1977; Lack 1968; Wittenberger and Tilson 1980). While valid, what contributes to a pair bonding relationship is often difficult to assess. Modern laboratory studies of pair bonding attempt to circumvent this criterion in an aim to understand the neurocorrelates of bonding by defining a pair bond through known species-specific bonding related behavior. Understanding the mechanisms governing bond related behaviors are not sufficient in understanding pair bonding as a whole. Pair bonding is a conglomeration of behaviors, each regulated by distinct neural regions and each having the potential of being performed without the creation of a bond. To date, no study has determined whether the brain encodes pair bonding as an entity or has attempted to empirically test which behaviors, if any, are associated with the formation of a pair bond.

There is no known phylogenetic pattern underlying the formation of a pair bond in animals. At least 3% of mammals (Kleiman 1977) and 90% of avian species (Lack 1968) are known to form pair bonds. Many theories have postulated why pair bonding has arisen in some species and not in others. Although these theories differ in terms of the exact purpose for the evolution of pair bonding, pair bonding is thought to have arisen in social species that would not be able to survive or produce offspring without the aid of a mate (Lack 1968; Kleiman 1977; Wittenberger and Tilson 1980). One of the more

recent theories as to why pair bonding has arisen, particularly in mammals, regards the dispersion of females within the group. If females are not aggregated, it becomes difficult for males to find females to mate with or to court multiple females at once (Lukas and Clutton-Brock 2013; Komers and Brotherton 1997). This suggests that pair bonding has arisen before the formation of social group, and pair bonding, therefore, may be a contributing factor to the evolution of the social group. This theory, however, is negated by some who believe that the formation of a pair bond followed the formation of social groups (Shultz et al 2011). The theory of dispersion as a limiting factor for mating is not a new one or a theory that has been limited to mammals. Birds of the same species have been found to take part in more extra pair copulations if the area they are in becomes more densely populated with females (Gowaty and Bridges 1991; Wagner 1993; Petrie and Kempenaers 1998). A comparative analysis across several avian species, however, suggests that there was no difference in extra pair copulations between animals in high and low density, which would be expected if density is a determinant of bond type (Westneat and Sherman 1997; Pietrie and Kempenaers 1998). It should be noted these studies did not distinguish between birds that formed bonds and those that did not. Most avian species are socially monogamous and partake in solitary nesting (Lack 1968) making comparisons between species in terms of bond formation difficult. Interestingly, other theories involving competition for access to mates have been proposed in regards to the evolution of pair bonding. Pair bonding may have evolved to aid in the avoidance of cuckolding by other males (Wittenberger and Tilson 1980) and to avoid injuries that could have resulted from competition to access for females (Chapais 2008; Chapais 2011). The evolution of pair bond formation has also been explained as a response to

selection pressure for paternal provisioning (Orians 1969; Lack 1968; Kleiman 1977; Wittenberger and Tilson 1980, Marlowe 2003, Marlowe 2001, Lovejoy 1981, Fraley et al 2005), overcoming diet constraints (Lack 1968; Wittenberger and Tilson 1980; Leonetti and Chabot-Hanowell 2011; Bell et al 2013), and the avoidance of predation and infanticide (Wittenberger and Tilson 1980; Shultz et al 2011; Orians 1969; Opie et al 2013; Quinlan and Quinlan 2007). It is important to note that pair bonding could have arisen distinctly in different classes or species and therefore the driving forces behind the evolution of pair bonding could differ depending on the animal being studied.

Due to its potential evolutionary link to parental investment, pair bonding is oftentimes associated with sexual behavior. Sexual preference or behavior and pair bonding, however, are not necessarily linked (Wickler 1976). An example of this can be seen in the reproductive strategy of marmosets (Callithrix jacchus). Marmosets were thought to be pair bonded due production of reproductive behavior being limited to the alpha male and alpha female of each group. The alpha male guards the alpha female from the sexual advances of other males during periods of estrus. The alpha female behaves aggressively towards other females, preventing them from having offspring of their own by inducing a stress response. Despite reproductive behavior being limited to one male and one female, this behavior was found to be a product of social ranking and not a bond because when the alpha females were removed from the group, the beta female took her place in the group (Rothe 1975). Rothe (1975) suggested that pair bonds have an emotional requirement that is not met by sexual behavior solely being directed at one animal. Kleiman (1977) later criticizes the "usefulness" of Rothe's suggestion, stating that, "mating exclusivity has genetic consequences, whereas an emotional bond (or lack

thereof) does not". Many socially monogamous animals, however, are not sexual monogamous and, therefore, the stated genetic consequences are attenuated by having sexual interactions and potentially offspring with other animals. Kleiman makes a valid argument when he questions the "usefulness" of a pair bond having an emotional requirement. An emotional requirement, while seeming like an obvious criteria for pair bonding, is not easily measured while sexual behavior is. Despite this, several criteria have been proposed to measure the attachment component attributed to bond formation. Attachment can be measured by assessing preference in a choice situation or the proximity to the object of attachment in free space (Wickler 1975). In order to determine if affiliative or reproductive behaviors lead to the formation of a bond, these behaviors must be empirically tested to determine if they meet the criteria used to measure attachment. In animals whose sexual exclusivity is determined by status aggression or other factors, such as the marmoset, pair bonding or attachment is much more difficult to ascertain since sexual preference may be due to other factors. Care should be taken to control for these factors to insure preference is due to attachment and not another extraneous variable (Wickler 1975). One way that this might be done is to determine if preference persists over time.

The most popular current animal model of pair bonding for human behavior (Young 2003; Young and Wang 2004), the prairie vole (Microtus ochrogaster) model, uses two bonding related behaviors, selective aggression and preference for a member of the opposite sex, to indicate the establishment and maintenance of a pair bond. These behaviors are usually initiated after voles are allowed to mate for a period of 6-24 hours, cohabitate for a period longer than 24 hours, or both (Williams et al 1992; Young 2003;

Winslow 1993; Aragona et al 2006; Aragona et al 2003; Wang et al 1999). Selective aggression is a species specific behavior that occurs post-mating in which prairie voles will attack members of the opposite sex they are not mated with. Both male and female voles partake in this behavior. Aggressive behavior towards unfamiliar members of the opposite sex does not occur in voles that have not mated or cohabitated (Getz et al 1981; Winslow et al 1993). Preference and selective aggression in voles, while different behaviors, seem to be linked. A vole that is selectively attacking members of the opposite sex, other than the animal they are mating with, will clearly maintain a preference towards that animal. This preference, however, will not be maintained if the voles are separated for a period greater than 24 hours (Getz et al 1981; Carter et al 1986).Partner preference in voles are thought to be induced by mating (Aragona et al 2003) despite cohabitation being used as an paradigm for bond formation, perhaps because sexual interactions between the vole speed up the onset of affiliative behaviors. Sexual behavior, however, need not occur for the onset of affiliative behaviors (Williams et al 1992), suggesting that copulation may not be necessary for the formation of a pair bond in voles. Alternatively, the act of cohabitating allows for the possibility of a number of behaviors to be performed between the male and female that are housed together, each of which may be contributing to partner preference, and therefore the formation of the attachment component necessary for a pair bond. These behaviors would have to be tested to determine which behavior leads to partner preference or attachment.

More interesting is that the endocrine and neurophysiological mechanisms regulating these bonding related behaviors (i.e. aggression in voles) have been selected as a model for pair bonding in humans. While selective aggression and cohabitation or

mating may be involved in pair bonding in voles, not all of these behaviors are present in humans. In a chapter entitled "The neural basis for bonding in a monogamous species: a model for understanding the biological basis of human behavior", Young states that there is no evidence that hormones involved in regulating bonding in voles are involved in human bonding (2003). Despite this, he mentions several studies connecting vasopressin, a hormone found to regulate bonding in voles, to sexual behavior and social attachment behavior unrelated to pair bonding (Young 2003). No study has empirically shown that sexual behavior is necessary for the formation or maintenance of a pair bond in humans. The findings of the vole studies have been a basis for understanding how fidelity is genetically regulated in humans (Cherkas 2004; Scheele 2012; Walum 2008). The results of these studies have been questioned because the genetic locus they targeted has been found to be stable across rodents, few of which are monogamous (Fink et al 2006). If variations in this gene were responsible for pair bonding in rodents or other mammals, you would expect polymorphisms within this locus to be present. Humans, like most socially monogamous mammals, are not sexually (genetically) monogamous (Reichard and Boesch 2003). A genetic component of fidelity in humans, therefore, is unfounded.

Functional properties of the amygdala and taeniae: emotional memories

Regulation of pair bonding would likely involve circuitry that has evolved early on and is present across a range of diverse species. Similarities in subcortical circuitry that are responsible for processing subconscious, emotional signals, across an array of species, including rats, birds, and humans, suggest that subcortical structures, such as the amygdala, might have evolved early on (Tamietto and de Gelder 2010). While behaviors

that govern partner preference may vary across species, all species that form pair bonds must be able to commit to memory who their mate is and must be able to distinguish their mate amongst a group of conspecifics. This mate representation may be mediated by the amygdala, a region of the brain known to be responsible for integrating emotional signals into memory. Perhaps because of ease of study, much of what is known about how emotional memories are processed via the amygdala is due to investigations into fear learning in mammals (Gallagher and Chiba 1996; McGaugh 2000; Davis and Whalen 2001; Phelps and LeDoux 2005; Dolcos et al 2004; LeDoux 2003; LaBar and Cabeza 2006; Hammann 2001; Richardson et al 2004). Despite this, the amygdala's precise role in the processing of emotional memories is debated (Maren 2003). In particular, it is disputed whether the amygdala is involved in the storage of emotional memory versus regulating memory process that occur elsewhere in the brain. While emotional stimuli are remembered more easily than neutral stimuli (Bradley et al 1992; Hamann et al 1999; LaBar and Cabeza 2006) and the amygdala has been demonstrated to be involved in regulating memory consolidation via other brain regions that are involved in memory processing (McGaugh et al 1996; McGaugh 2000; Dolcos et al 2004), there are several studies that suggest that it may have more than one function. Inactivation of the amygdala using GABA agonists prior to a fear learning paradigm resulted in rats showing significantly less of a fear response, mainly freezing response, than those that were treated with a vehicle (Muller et al 1997; Helmstetter and Bellogowan 1994; LeDoux 2003) suggesting that the amygdala, specifically the basolateral nucleus, plays a role beyond that of regulating memory consolidation. Additionally, several studies have pointed to different nuclei of the amygdala, mainly the central nucleus and the basolateral

nucleus, having key, but distinct, roles in the processing of fear with the basolateral nucleus mainly being responsible for mediating fear conditioning while the central nucleus is mainly responsible for the expression of fear responses via its connectivity with brain regions involved in motor behavior (Davis and Whalen 2001; LeDoux 2003; Maren 1999; Gallagher and Chiba 1996).

While not as well studied, the amygdala has also been implicated in positive, or appetitive, emotional memories. Arousing memories are remembered more readily than non-arousing memories regardless of their valance. When subjects were shown pictures of both appetitive and aversive stimuli, they were more readily remembered if the stimuli were more arousing. It did not make a difference if the image was of a positive or negative nature (Bradley et al 1992). Furthermore, amygdala activation is correlated with enhanced recognition of both appetitive (positive) and aversive (negative) stimuli, suggesting that the amygdala's function is subject to level of emotional arousal and not stimulus valence (Hamann et al 1999). Stimuli that were classified as unusual also activated the amygdala, suggesting the amygdala also has a role in assessing ambiguous stimuli (Hamann 2002). Involvement of the amygdala in appetitive learning is a bit more complex, however. Appetitive stimuli, such as food, has its own intrinsic value representation such that lesions to the amygdala have no effect on food preference or stimulus-reward processing. The amygdala, in particular the basolateral nucleus, however, has a role in updating emotional information from the environment. If an animal is allowed to eat a particular food ad libitum until satiety and, afterwards, it is given the choice between that food item and another food item, it will choose the other food item regardless of initial preference. Lesions to that amygdala destroy the effect of

satiety on food choice (Baxter and Murray 2002; Everitt et al 2003). Animals that have basolateral nucleus lesions cannot perform second-order conditioning tasks (tasks in which a second conditioned stimulus is associated with the affective value of the first conditioned stimulus) and lose specificity in terms of pairing particular instrumental responses to their conditioned stimuli, consistent with the idea that they are unable to assess the updated emotional value of stimuli (Everitt et al 2003). Similar to the central nucleus' role in fear learning, in stimulus-reward learning this nucleus functions in mediating response to the conditioned stimulus. Lesions to the central nucleus eliminate "autoshaping behavior", a behavior seen in several species including primates, humans and rats, in which an animal approaches a conditioned stimulus (Parkinson et al 1999; Baxter and Murray 2002; Everitt et al 2003; Murray 2007).

In avian species, the structurally homologous region to the mammalian amygdala is called the nucleus taeniae of the amygdala (Cheng et al 1999; Reiner 2004). While much is not known about the functional role of the taeniae, several studies suggest that it has a similar role to its mammalian counterpart in integrating emotionally relevant stimuli. Like the mammalian amygdala, the nucleus taeniae has been shown to be involved in the processing of aversive stimuli. Pigeons who were trained via tone-shock conditioning developed a freezing response much like those that were continuously shocked, while pigeons in a tone-only group had an initial freezing response that was attenuated after several trials and those in the control group had no freezing response. Pigeons in the tone-only group, shock group, and tone-shock group had greater amounts of ZENK labeled cells in the nucleus taeniae in response to their group condition (Brito et al 2011). A neuroimaging study looking at a crow's perception of human faces showed

taeniae activation in response to the face of their captor, suggesting that this area may be involved in the perception of fearful stimuli (Marzluff et al 2012).

Studies in rats and hamsters have demonstrated that the amygdala, specifically to the medial nucleus, has a role in sexual behavior (Harris and Sachs 1975; Kondo and Yamanouchi 1995; Wood and Newman 1995). Testosterone implants into the medial nucleus and BNST/MPOA have been found to increase bouts of sexual behavior suggesting that steroid receptors in these regions function in mediating sexual behavior in hamsters (Wood and Newman 1995). Lesions to the corticomedial and medial nucleus of the amygdala have been shown to decrease sexual behavior (Harris and Sachs 1975; Kondo and Yamanouchi 1995). Bilateral lesions to the nucleus taeniae in quail have yielded mixed results (Thompson et al 1998; Absil et al 2002). One study that bilaterally lesioned the nucleus taeniae in male quail reported a significantly longer latency in approaching stimulus females, significantly less foam gland movements towards females, and a marginally significant effect of less time spent in proximity to a sexually-conditioned object (Thompson et al 1998). A second study found that taeniae lesions had no effect on look response times and rhythmic cloacal sphincter movements in the presence of females, measures of appetitive or preparatory sexual behavior, but had significant increases in mount attempts and cloacal contact movements, a measurement of consumatory or sexual performance behavior (Absil et al 2002). The authors attributed this to differences in lesion sites. The lesions in the first study were more posterior. Birds can detect changes due to taeniae lesions (Ikebuchi et al 2009). Female zebra finch will not choose males with taeniae lesions during preference tests and lesioned males will not sing during tests. The lesioned male's ability to sing, however, is not altered. When

males and females are removed from the preference test setting and lesioned males are allowed to interact with females without the presence of another male, they sing. Additionally, females will accept the males during this interaction and engage in courtship behaviors such as clumping. This suggests that taeniae lesions do not destroy motor behavior, but instead may be involved in regulating social behavior, such as status perception (Ikebuchi et al 2009). Bilateral lesions in ring doves increase nest coo behavior, a type of courtship behavior, confirming these results (Cheng et al 1999). The taeniae develops early ontologically, in pace with sensory areas, suggesting that it may be mediating early social behaviors (Ikebuchi et al 2013). In zebra finch, pair bonding related behaviors, mainly allopreening and clumping, increased the amount of immediate early gene ZENK in the nucleus taeniae, supporting the idea that the taeniae may function in processing socially relevant stimuli (Svec et al 2009).

Human studies suggest that the amygdala may function in, not just the processing of emotional memories, but also in the perception of emotional stimuli. The amygdala has been shown to be activated when humans view images emotional facial expressions (Morris 1998; Hariri et al 2002; Haxby et al 2002), suggesting that the amygdala may play a role in perception of emotionally salient cues. Individuals with deficits in reading emotional cues, such as individuals with social phobias (Stein 2002; Niels et al 1998; Phan et al 2006) or autism (Shultz 2005; Grelotti et al 2005) differ from controls in amygdala activation. Animal studies validate these findings. Damage to the medial amygdala in hamsters has been shown to disrupt preference for mates of the opposite sex, but not memory of the individual itself, suggesting that the amygdala may play a role in preference, but not memory of an individual (Pertulis and Johnston 1999). Follow up

studies in hamsters have found that the medial amygdala is involved in discriminating conspecifics versus heterospecifics (delBarco-Trillo et al 2009) suggesting that the medial amygdala may play a role in more than just preference. Additionally, only the medial amygdala was damaged in these studies, allowing for the possibility that other areas of the amygdala may function in the discrimination of individuals. Pertulis speculates that the cortical amygdala may be one of the neural substrates that is involved in the recognition of individuals (Petrulis 2009). Whether the amygdala's role in pair bonding involves the perception of emotionally salient cues received by the subject after exposure to its mate or the animal having a memory for its mate may be difficult to assess. We suspect that the amygdala is involved in retaining the memory of the mate because, if the amygdala is involved only in the perception of emotional stimuli, the amygdala would respond equally to any member of the opposite sex, as they are perceptually similar, rather than specifically towards the mate (a criterion that is needed for pair bonding).

Amygdala and taeniae connectivity:

The mammalian amygdala is an almond shaped region in the temporal lobe that is composed of several interconnected nuclei (Pitkanen et al 1997; Sah et al 2003; Swanson and Petrovich 1998). Besides its role in the functions described above, the amygdala also has a role in attention, perception of body movements, and generation of emotional aspects of dreams (Pitkanen et al 1997). Although there is some debate as to which nuclei compose the mammalian amygdala and how they should be subdivided, connectivity and functional studies between nuclei have helped to validate the currently

recognized subregions (Krettek and Price 1978; Pitkanen et al 1997; Amaral et al 1992). Three key features of the amygdala can be seen across several mammalian species including rats, cats, and monkeys. Mainly, there is sensory input into the amygdala and brain areas that are involved in this input often receive efferent projections back from the amygdala, there are efferent projections to both the hypothalamus and brain stem, and there are efferent projections to forebrain regions that are involved in mood (Price 2003).

For the purpose of simplicity, anatomical features of the mammalian amygdala will be described in rats. Briefly, the nuclei of the rat amygdala are: the *lateral nucleus* and *basal nucleus* (which are sometimes grouped together), *accessory basal nucleus*, *central nucleus*, *medial nucleus*, *anterior amygdaloid area*, *amygdalo-hippocampal area*, *nucleus of the lateral olfactory tract*, *bed nucleus of the accessory olfactory tract*, *anterior cortical nucleus*, *periamygdaloid cortex*, *posterior cortical nucleus* (Pitkanen et al 1997). Several efferent projections have been identified in the rat amygdala (See table 1). The medial subdivision of the *central nucleus* projects to dorsolateral and caudolateral regions of the *hypothalamus*, the *bed nucleus of stria terminalis*, the *periaqueductal gray*, the *parabrachial nucleus*, and the *nucleus of the solitary tract*. More generally the *central nucleus* projects to the *bed nucleus of the stria terminalis*, which functions to innervate several *hypothalmatic nuclei*, *locus coeruleus*, *substancia nigra*, *ventral tegmental area*, *raphae*, and *nucleus basilis*. The *medial nucleus* sends projections to several *hypothalmatic* regions including the *ventral medial nucleus* and the *anterior paraventricular nucleus*. The *medial nucleus* also sends projections to the *perirhinal area*. The *basolateral nuclei* also send projections to the *perirhinal area* as well as to *the nucleus accumbens* and *hippocampus*. The *posterior basal nucleus* sends projections to *ventromedial nucleus of the hypothalamus*. The *basal nucleus* projects to the *prefrontal cortex*. The *periamygdaloid cortex* sends projections to

the *perirhinal area*. The *cortical nucleus* returns projections it receives from the *olfactory cortex* (Sah et al 2003; Swanson and Petrovich 1998).

The rat amygdala receives sensory inputs from various regions that mediate the different sensory modalities. Projections coming from olfactory inputs include those from the *accessory olfactory bulb*, which projects to the *bed nucleus of the olfactory tract*, *the medial nucleus*, and the *posteriormedial cortical nucleus*; the *piriform cortex* and *anterior olfactory nucleus*, which project to the *basalateral nuclei*; the *doral endopiriform nuclei*, which project to all *cortical nuclei of the amygdala*, the *nucleus of the lateral olfactory tract*, the *periamygdaloid cortex*, and the *medial nucleus*; and the *main olfactory bulb*, which projects to the *main olfactory tract*, the *anterior cortical nucleus*, the *posteriorlateral cortical nucleus*, and the *periamygdaloid cortex*. The *main olfactory bulb* also projects to the *piriform-amygdalar area* which projects to several *amygdala subregions*. Auditory input comes from *Te3* (secondary auditory area) and the *medial geniculate nucleus* which have projections to the *lateral nucleus*. Visual input is received from *Oc2* (visual cortex) via Te2 (visual association cortex) an projected to *dorsolateral subdivsions of the lateral nucleus*, the *basal nucleus*, and the *lateral division of the central nucleus*. Somatosensory inputs are received via the *dysgranular parietal insular cortex*, which projects to the *dorsolateral region of the lateral nucleus*, the *basal nucleus*, and the *central nucleus*; the *posterior internuclear nucleus* sends projections to the *lateral nucleus*, the *accessory basal nucleus*, the *medial division of the central nucleus*; the *medial portion of the medial geniculate nucleus* and the *pontine parabrachial nucleus* also sends somatosensory input to the *amygdala*. Visceral sensory information from the *posteriormedial thalamic nucleus* is sent to the *lateral nucleus*, the *basal nucleus*, and the *central nucleus*; the *parabrachial nucleus* sends projections to the *lateral division of the central nucleus* and and the *insula* also sends input to the *lateral nucleus*, the *basal nucleus*,

and the *central nucleus*. Several sensory inputs which function across several sensory modalities also send projections to the *amygdala* including the *prefrontal cortex*, *perirhinal cortex*, *hippocampus*, and *entorhinal cortex* (Sah et al 2003; Swanson and Petrovich 1998).

The only anatomical study assessing taeniae connectivity in birds has been done in ring doves, Streptopelia risoria, and European starlings, Sturnus vulgaris (Cheng et al 1999). Although there are some small anatomical differences between starlings and ring doves as a consequence of the starling's larger forebrain, connectivity seems to be conserved between both species (See table 1).

In ring doves and starlings, the taeniae occupies the ventral border of the archistriatum. This region contains two major ascending and descending pathways. The *first descending pathway* was termed *hypothalmatic-occipitomescenphalic tract*. Briefly, fiber bundles originating in the *taeniae* bilaterally project to the *bed nucleus of stria terminalis lateralis* and through the *occipitomescenphalic track* and some fibers reach *periventricularis magnocellularis*, *preoptic anterior medial hypothamaus*, and *posterior medial hypothalamus*. Other fibers terminate in the *medial septum*, *shell region of the nucleus ovoidalis*, and *anterior commissure*. Another *descending pathway* was present in the *midbrain*, where fibers were found in the *nucleus intercollicularis* and the *nucleus lemnisci lateralis pars dorsalis*. An *ascending pathway* runs from *bed nucleus of stria terminalus* and along the *lateral wall of the lateral ventricle* with fibers along the way terminating on the border of the *lamina hyperstriatic* and *lamina medullaris dorsalis* and the *medial region of the parolfactoris* .

Table 1: Amygdala and taeniae connectivity in birds and mammals

	Efferent	**Afferent**
Mammalian (rat)	hypothalamus BNST pons • locus coeruleus • parabrachial nucleus midbrain • substancia nigra • ventral tegmental area • raphae • periaqueductal gray basal forebrain • nucleus basilis • nucleus accumbens medulla • nucleus of solitary tract hippocampus perirhinal area pre-frontal cortex olfactory cortex	Olfactory regions • olfactory bulb • accessory olfactory bulb • anterior olfactory nucleus piriform cortex (including endopiriform nucleus) Auditory regions • auditory cortex (Te3) • thalamus (medial geniculate nucleus) Visual regions • visual cortex (Oc2) • visual association cortex (Te2) Somatosensory regions • insula (dysgranular parital insular cortex) • thalamus (medial geniculate nucleus) • pons (posterior internuclear nucleus, pontine parabrachial nucleus) Visceral regions • thalamus (posterior medial thalamic nucleus) • pons (parabrachial nucleus) • insula Multiple sensory inputs: • prefrontal cortex • perirhinal cortex • hippocampus • entorhinal cortex
Avian (ring doves)	BNST hypothalamus Olfactory regions • medial septum thalamus • shell region of the nucleus ovoidalis midbrain • nucleus intercollicularis • nucleus lemnisci lateralis pars dorsalis	BNST hypothalamus Homologous to mammalian pallial regions • lamina hyperstriatic • ventral hyperstriatum • archistriatum basal forebrain • nucleus accumbens hippocampus parahippocampal area Olfactory regions • medial region of the paraolfactoris • lateral septum • medial septum • olfactory bulb thalamus • shell region of the nucleus ovoidalis (auditory) • subrotunal nucleus (visual) • lamina medullaris dorsalis pons • locus coeruleus Homologous to mammalian pyramidal tract • septomesencephalius (TSM)

The second major *ascending pathway* runs from the *medial septum* along the *medial wall of the lateral ventricle* and terminating in the *hippocampus*, and in the *parahippocampal area*. *Afferent projections* to the *nucleus taeniae* were found in the *shell region of the nucleus ovoidalis* (avian auditory thalamus), along the *ventral hyperstriatum*, in the *olfactory bulb*, in the *subrotundal nucleus*(avian visual thalamus), the *parahippocampal area*, *anterior archistriatum*, *nucleus accumbens*, the *lateral septum*, *tractus septomesencephalicus*, *the preoptic area*, *dorsolateral hippocampus* and *area parahippocampus*, and *locus ceruleus* (Cheng et al 1999).

While subregions of the mammalian amygdala have been delineated via anatomical studies, no study has assessed this in the nucleus taeniae. The taeniea is oftentimes equated with the mammalian medial amygdala due to its afferent projection from the olfactory bulb (Cheng et al 1999; Reiner et al 1985; Patzke et al 2011) and its efferent projection to the hypothalamus via the hypothalmatic-occipitomescenphalic tract (Cheng et al 1999), as well as behavioral studies that have suggested that the taeniae has a role in sexual behavior (Thompson et al 1998; Absil et al 2002; Ikebuchi et al 2009), a role that is also associated with the medial amygdala in mammals (Newman 1999). While accurate, the taeniae has a role in other behaviors, such as fear conditioning (Brito et al 2011), that requires the integration of multiple nuceli within that mammalian amygdala. Additionally, the nucleus taeniae contains projections to areas that are outside those found in the medial nucleus, but can be seen in a variety of other amygdala nuclei. Further studies aimed at assessing whether there are amygdaloid subregions within the avian brain subject to their own distinguishing anatomical and functional features must be done in order to clarify this matter.

Reproductive behavior in doves:

Pair bonding is a complex behavior that contains an emotional component which leads to preference, but may behaviorally manifest itself differently across different species. Understanding this emotional bond, therefore, requires studying a species that not only forms a pair bond, but that has reproductive and courtship behavior that is well established. Ring doves, *Streptopelia risoria* engage in a predictable courtship cycle in which hormonal and neuronal components have been extensively studied (Wallace 1908; Miller and Miller 1958; Lovori and Hutchinson 1975; Lehrman 1964; Lehrman 1959; Cheng 1979). The courtship cycle in doves begins with a greeting behavior called the bow coo (Miller and Miller 1958). The exact significance of the bow coo is not known, however, the bow coo is testosterone dependent (Klint et al 1984) and only performed by males and the bow coo of each male differs in several characteristics, suggesting that the bow coo may not only communicate the gender of the bird, but also may serve as a further identifier for that specific male (Fusani et al 1997). Following the bow coo, male doves will partake in a courtship coo called the nest coo. The nest coo is performed simultaneously with wing flipping behavior, a quick jerking wing motion (Miller and Miller 1958). Although the males will initiate nest coo behavior, receptive females eventually will participate at a prospective nest site. Around this time, the females will engage in crouching behavior signaling that they are ready to copulate. Males will generally not attempt copulation the first few times the female engages in crouching behavior, but will eventually mount and copulate with the female (Miller and Miller 1958; Lovari and Hutchinson 1975). While doves copulate during earlier stages of the courtship routine, these copulations do not lead to fertilization as the hormonal state

necessary for ovulation hasn't been reached at this time (Cheng et al 1981). Nest coo behavior will gradually lessen and eventually cease in preparation for ovulation, incubation, and squab rearing behaviors (Lehrman 1964; Silver 1978). Both visual and auditory aspects of the coo guide the courtship routine. While deaf males are still able to perform courtship behavior, the components of the bow coo differ from that of a non-deafened male (Nottebohm and Nottebohm 1971). In females, deafening leads to a delay from the initiation of the courtship routine to ovulation (Nottebohm and Nottebohm 1971). Vocalizations alone, however, are not sufficient in enlisting females to partake in courtship; a visual stimulus is also needed for this to occur (Lambe and Erickson 1973). Visual and auditory components directed towards the female aid in her engagement in courtship behavior (Friedman 1977). Female doves will lay two eggs following this courtship routine and nest-building phase and these eggs will be incubated by both the male and female dove of the pair (Lehrman 1964; Lehrman 1959). For males, previous exposure to the female during initial stages of the courtship routine is necessary for incubation (Silver et al 1973; Lehrman 1958). Generally, males tend to incubate eggs during the middle of the day, while females incubate for the remainder of the time (Lehrman 1964; Lehrman 1959). Males will continue to incubate if the female is removed, despite a novel female being introduced, if they have gone through earlier parts of the courtship cycle with their mate (Silver and Barbiere 1977). Several days (3-4) before the hatching, the crops of both males and females begin to grow in preparation for feeding the squab. Once the eggs hatch both the male and female dove feed the squab until they are old enough to provision for themselves (Friedman and Lehrman 1968; Cheng 1979).

The courtship cycle in ring doves is regulated by hormones. In male doves, testosterone plays a role in bow coo behavior, but not nest-solicitation (nest coo) behavior, and estradiol increases the amount of nest-solicitation behavior (Klint et al 1984; Hutchison 1970). Incubation in males is aided by progesterone (Lehrman 1958(2)), but progesterone does not seem to be necessary for incubation behavior to occur in experienced pairs (Cheng et al 1986; Silver and Buntin 1973). Cheng found that plasma progesterone facilitated behaviors prior to incubation, shortening the latency to incubation (1975). Therefore, it seems that once incubation behavior has commenced, progesterone may not be needed for it to continue. In males, crop size increases due to prolactin several days before the young hatch (Riddle and Dykshorn 1932) but this occurs only if the male associates with the female during the courtship stage (Friedman and Lehrman 1968). Prolactin levels are maintained after the hatching of the squab until the squab are mature enough to feed themselves. At this point prolactin levels decrease and the cycle restarts (Cheng and Burke 1983; Wortis 1969; Cheng 1979).

It was originally thought that the male's activity during the courtship cycle directly induced ovarian activity in females (Erickson 1970; Barfield 1971; Cheng 1992). This view was contradicted by a series of experiments suggesting that the female's own nest coo, stimulated by the male's courtship, is responsible for inducing the female into the appropriate hormonal state necessary for her own reproductive cycle (Cheng 1992). Specifically, it is thought that the female's own nest building activity stimulates the FSH decrease and LH surge that is characteristic of ovulation (Cheng 1992; Cheng and Balthazart 1982). Devocalizing the female, which produces a clear block in the female's nest cooing behavior (despite a male courting her) will prevent follicular development

(Cheng 1986; Cohen and Cheng 1979; Cohen and Cheng 1981; Cheng 1992). When devocalized females listened to nest-coo play backs, they had greater follicular growth when they listened to their own coo's as compared to that of another female or males (Cheng et al 1988; Cheng 1992). Electrophysiological recordings from cells in the hypothalamus identified coo sensitive neurons that respond to female coos, but not playbacks of reversed nest coos, by releasing LH in preparation for ovulation (Cheng et al 1998). Cells in the auditory thalamus and midbrain auditory nucleus selectively respond to nest coo behavior and some cells in the auditory thalamus that respond to nest coo, selectively do so to male coos specifically (Cheng and Havens 1993; Cheng 1992). The midbrain auditory nucleus and the auditory thalamus have projections that terminate in the hypothalamus suggesting a route for the nest-coo's role in mediating follicular growth (Cheng and Zuo 1994; Cheng 1992; Gibson and Cheng 1979; Bernstein et al 1993). Since these studies, it has been accepted that the female plays a role in her own activity by evaluating a potential mate and going through the process necessary for self-stimulation (Cheng 2008).

While courtship behavior in females is regulated by estrogen, (Cheng and Silver 1975), estrogen at high levels reverses this effect (Cheng 1973). LH-RH allows females at a high estrogen threshold to court suggesting that it has a role in courtship behavior (Cheng 1977). It must be noted that LH-RH administered alone will not produce courtship behavior suggesting that it functions in conjunction with estrogen (Cheng 1977). Estrogen in females peaks during ovulation, but declines during incubation (Korenbrot et al 1974). Nest building in females is governed by progesterone with females showing a sharp rise in progesterone around the time of egg laying (Cheng and

Balthazart 1982; Cheng 1979). Like with male doves, prolactin is secreted during incubation in females in order to aid in crop growth and the production of crop milk (Cheng and Burke 1983; Erickson and Lehrman 1964). Prolactin is also present earlier during incubation and functions in lowering the gonadal hormones (estrogen and progesterone) in order to aid in incubation behavior (Cheng 1979).

Morris and Erickson found that males who went through a breeding cycle and were separated from their mate for up to a period of seven months were able to distinguish the female which they were mated to amongst a group of other females (1971). Males were found to spend more time in proximity to their mates when housed in an enclosure that contained other females and preferentially performed higher amounts of courtship behavior towards their mates in a preference paradigm after separation (Morris and Erickson 1971), a mark of the emotional bond necessary for bonding. The motor behavior involved in the courtship routine isn't sufficient to form a bond, it must be tied with a particular female. Although the courtship routine is predictable and follows the specific order detailed above, if a male that has undergone part of the courtship routine is introduced to a novel female, the male will not resume courting, but will instead begin the courtship routine from the beginning of the cycle (Silver et al 1973; Silver 1978; Silver and Barbiere 1977). Males who encounter their mate with another male will aggressively attack the female if she is ovulating (Zenone et al 1979; Erickson 1986). Males are more aggressive to females that have been pre-exposed to other males and therefore are past the initial phase of the courtship routine, despite themselves being pre-exposed to match the physiological state of the female (Rissman 1983; Erickson 1986). The interaction of males and females during the courtship (pre-laying) portion of the

breeding cycle has been found to stimulate GnRH labeled newborn cells that lead to an increase in the total number of GnRH cells in the ventromedial nucleus of the hypothalamus (VMN) which is responsible for the regulation of reproductive behavior in doves (Cheng et al 2011).

Given that the reproductive cycle in ring doves has been extensively studied and that the hormonal regulation and circuitry underlying courtship and reproductive behavior have been well established, they are an ideal animal species to examine how pair bonds are formed and governed and to assess the impact of pair bonding on survival related behaviors. The aim of my thesis is to investigate the neurophysiological regulation of pair bonding. More precisely, my thesis aims to determine what brain regions, if any, are involved in representing pair bonding. In particular, due to its ideal connectivity and function in processing emotional stimuli, we seek to determine whether the nucleus taeniae is responsible for regulating the formation and maintenance of a pair bond. In order to understand the neurophysiological substrates governing pair bonding, we must first understand the phenomenon of bonding itself. Pair bonding has been found to be initiated by completing the breeding cycle. Doves that go through a complete breeding cycle, including the rearing of a squab retain preference, or attachment, to their mate despite a period of separation (Morris and Erickson 1971). To determine when during the breeding cycle pair bonding occurs, we will examine various phases of the breeding cycle including pre-egg laying courtship behavior, egg laying, and squab rearing. Additionally, we will assess whether the nucleus accumbens, an area found to mediate rewards processes for reproductive behaviors in other species, is associated with pair bonding in ring doves. The taeniae's large fiber projection to the hypothalamus allows for potential

moderation of reproductive behavior, including courtship behavior, in ring doves. Since pair bonding is an important survival strategy in adult doves, we aim to determine whether pair bonding status would aid in the increased production of differentiated new neurons in the hypothalamus (the VMN) and recovery of courtship (nest coo) behavior after lesions to this region.

Chapter 1

Study 1: Is there a neural marker for pair bonding?

As published: Dios et al 2013 with addendum for additional analysis and experiment

Introduction:

Pair bonding is of importance in understanding relationship dynamics between males and females within a given social species. Aside from being involved in aiding in male parental care (Mock and Fujioka 1990), a pair bond functions in insuring paternity (Mock and Fujioka 1990) and aiding in provisioning (Marlow 2003, Quinlan and Quinlan 2007, Schwagmeyer and Mock 2003). Separation from a mate pair has been shown to lead to weight increase and a diminishing of social interaction and exploratory behavior in hamsters (Crawley 1984).

Monogamous pair bonded species have shown that they have been known to stray. The monogamous bank swallow, which forms lifetime pair bonds, has been seen spending time alternating between mate guarding and trying to seek out extra pair courting opportunities (Beecher et al 1979). The right balance of these two alternating behaviors is said to be associated with producing as many offspring as possible while insuring that its mate's offspring are also his own (Barash and Lipton 2001; 30).Straying has been known to increase reproductive success in males (Wetton et al 1995, Forstmeier 2002) and females (Dunn et al. 1994, Lubjuhn et al. 1999; Forstmeier 2002; Gray 1997, Kempenaers 1992). Paternity tests in many avian species that were once thought to be

monogamous now show that this is not the case (Birkhead et. al 1990, Wetton 1995, Griffith et. al 2002). Eggs from the nests of monogamous birds have a high percentage of differing paternal DNA (Birkhead et. al 1990, Griffith et. al 2002).

Life-long pair bonds are seen in animals that seek out their mate amongst a group of conspecifics despite an extended amount of separation. In a study done by Morris and Erickson (1971), doves that mated and reared a squab together exhibited this mate seeking behavior in an outdoor arena, amongst a large group of other doves, despite being separated from each other for over a year. In the laboratory, triad tests are used to mimic this effect by using amount of time spent on sides of a preference chamber to look at a subject's preference to spend time with their mate versus a stimulus. They serve as a proxy measurement of pair bonding in which animals that spend more time with their mate are considered to be pair bonded. We reason that a neural marker, if such a marker exists, may be a more reliable indicator of pair bonding, would establish that there is a neural representation for pair bonding in the brain, and may solidify the importance of pair bonding as a deep rooted, evolutionary driven behavior. Currently, no study has established that there is a neural representation for pair bonding in the brain. Previous work done in zebra finch has implicated the nucleus taeniae (avian amygdala) in having a role in preening and clumping, two behaviors that are associated with courtship in finch (Svec et al 2009). Studies in hamsters, rats, and quail have shown that areas of the amygdala/nucleus taeniae are responsible for the mediation of sexual satiety, appetitive behavior, and consummatory behavior (Absil 2002; Parfitt and Newman 1998).

Tract tracing work done in the nucleus taeniae has shown that it has afferent and efferent connections towards several hypothalmatic regions and reward system areas, including the nucleus accumbens (Cheng et al 1999). These connections, as well as the studies mentioned above, suggest that the nucleus taneiae may be a likely candidate in encoding stimulus properties leading to pair bonding, might serve as a neural marker for such behavior, and could function in mediating pair bonding behavior in the brain.

Studies in rats, birds, and humans have shown that there is a similarity in subcortical circuitry involved in unconscious, emotional signals and suggests that subcortical structures, such as the amygdala, involved in processing emotional signals evolved early on (Tamietto and Gelder 2010). By understanding behaviors, such as pair bonding, controlled by these subcortical, reward driven regions, we might begin to understand pair bonding behavior across multiple species.

The present study asks if ZENK expression in the nucleus taeniae is a reliable indicator of pair bonding, therefore having the ability to serve as a neural marker for pair bonding. A quadratic discriminant analysis, which takes into account regional specificity within the nucleus taeniae, is used to classify doves as either pair bonded or not bonded.

In Experiment 1, we looked at whether ZENK counts in the taeniae were able to classify both male and female doves as pair bonded or not. In Experiment 2, we assessed if this classification was persistent when using a larger, all-female group. In both experiments, doves are tested using a preference test to determine the reliability of traditional stimulus preference tests in classifying pair bonded doves.

Methods:

Experiment 1:

The subjects were a group of ten ring doves bred and housed in an animal housing facility in the Association for Assessment and Accreditation of Animal Care (AAALAC) accredited animal care facility at Newark, Rutgers University. The doves were equally divided into two groups: a bonded group (n=5; 3 females and 2 males) and a non-bonded group (n=5, 5 females). The bonded group was allowed to mate with a female (if male) and one male (if female) and rear at least one squab, as described by Morris and Erickson (1971). The non-bonded group and bonded group were housed separately a week prior to the initiation of the experiment. During the experiment, the non-bonded group was housed with stimulus males that were rotated out of their cage on Monday, Wednesdays, and Fridays of every week in order to prevent the possibility of them bonding.

Stimulus Preference Test:

Doves in both groups were put through a y-shaped preference test developed by our laboratory (Clavijo and Cheng 2007) (Figure 1). The male dove was allowed to roam the chamber for 15 minutes preceding data collection in order to get acclimated with the chamber.

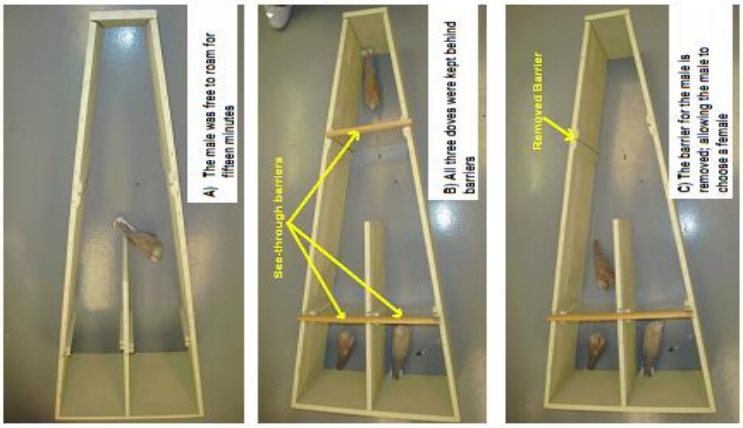
Afterwards, two females (if subject was male) or two males (if subject was female) were put into plexi-glass contained chambers opposite each other. The males in the pair bonded group were exposed to their mate and a novel stimulus female (if male) or a novel stimulus male (if female) while the birds in the non-pair bonded group were

exposed to two novel stimulus males. The subject was put behind a plexi glass compartment that allowed it to view both females (if male) or both males (if female) but not interact with them or roam through the chamber. It was held there for an additional 15 minutes.

The plexi-glass divider containing the subject in its chamber was then lifted and the subject was allowed to roam the chamber freely for an hour. During this time, we recorded the amount of time spent on each side of the preference container.

A percentage of time spent with their mate was calculated by dividing total time spent with mate by total time spent with the stimulus bird and mate. Birds were determined to have a preference for their mate if they spent more than 60% amount of time with their mate.

Figure 1: Preference Test



Immediate early gene- ZENK staining:

Subjects in both groups were perfused using a 4% paraformaldehyde solution. Brains were extracted and fixed in 4% paraformaldehyde solution overnight. Tissue was stored in a 30% sucrose solution for approximately two days post fixation.

Tissue was cut into 30 micron coronal sections, washed, and stained in accordance to procedures described by Svec et al (2009). Briefly, tissue was washed and incubated in a 1ug/ml dilution of primary antibody anti-Egr-1 (Santa Cruz Biotechnology Inc, Dallas, TX Catalog # sc-189) overnight. The tissue was then rinsed and incubated in a 1:500 dilution of secondary Biotin-sp-conjugated donkey anti-rabbit IgG antibody (Jackson Immuno Research Laboratories, Inc, West Grove, Pennsylvania, catalog #712-065-153) for 1 hour. Post-incubation, tissues were processed using Vectastain ABC Elite Kit (Vector Laboratories, Burlingame, California, catalogue #PK-6100) followed by visualization using diaminobenzidine with a 0.0075% hydrogen peroxide. Tissue was then mounted, dehydrated, and coverslipped. Control sections without primary antibody also underwent this process.

Statistical analysis and ZENK quantification:

For each bird, three sections (sampled from a rectangular area measuring 105um x 190um at 20x magnification) were counted and averaged for both medial and lateral portions of each of the following regions of the nucleus taeniae corresponding to the pigeon brain atlas (Karten and Hodos 1967): 7.50-7.25 region (more anterior); 7.25-7.00

region; and 6.75-6.50 region (more posterior). The most anterior sections (beginning with the 7.5 region) were landmarked by the appearance of the tractus occipitomesencephalicus (OM). Medial regions of the anterior portions of the taeniae were laterally boarding OM. Lateral portions of the anterior taeniae are bordered by the ventral archistriatum. Cells in the medial and lateral portions of the taeniae appeared to differ. Cells in the medial portions were smaller and this area was more densely populated with cells than lateral portions where cells appeared to be larger and more spread out. In posterior regions of the taeniae, the OM landmark could no longer be seen. Posterior taeniae was bordered dorsally by the ventral archistriatum. The posterior taeniae is bordered medially by the nucleus tractus septomesencephalici (SPC) and the tractus opticus (TrO). The taeniae reached its most posterior boundary before the appearance of the tectum opticum (TeO) (region 6.25). T-tests were done to determine if there were differences in cell counts between bonded and non-bonded doves for these regions.

Classifier analyses have been shown to be a more rigorous measure of how the brain identifies an object because it allows for cross-validation (Hanson and Halchencko 2008). We sought to assess whether a brain region, the nucleus taeniae, could accurately represent a state of the bird, in this case, whether it was pair bonded or not, following exposure to a female (either bonded or non-bonded, depending on the group they were in). We, therefore, used a linear and quadratic discriminant analysis (types of classifier analyses) to determine whether we could classify doves via their bond status by using ZENK cells in the nucleus taeniae. First, a principal component analysis was conducted on the ZENK cell counts in order to reduce dimensions. We then trained classifiers on

principal component scores allowing us to predict which doves were bonded versus non-bonded based on these scores.

Experiment 2:

This experiment was carried out to replicate the first experiment and account for variance due to differences in sex of the subject pool. In the first experiment, we used a small sample number, but subjects were of varying sex. Our current experiment was carried out in a group of females.

The subjects were a group of sixteen female ring doves bred and housed in an animal housing facility in the AAALAC accredited animal care facility at Newark, Rutgers University. The doves were equally divided into two groups: a bonded group (n=10) and a non-bonded group (n=10). As in Experiment 1, the bonded group consisted of females that were allowed to go through the bonding process and were housed separately a week prior to the initiation of the experiment. The non-bonded group was housed with stimulus males that were rotated out of their cage on the Monday, Wednesday, and Friday of every week in order to prevent the possibility of them bonding.

Male Subject group:

An additional group of five pair bonded males were allowed to undergo the same procedure as their female counterparts. A t-test was done on ZENK cell counts to determine if there was a difference between male and female subjects.

Stimulus Preference Test:

The same protocol was used as in Experiment 1.

Immediate early gene- ZENK staining:

Similar to Experiment 1, subjects in both groups were perfused using a 4% paraformaldehyde solution. Brains were extracted and fixed in 4% paraformaldehyde solution overnight. Tissue was stored in a 30% sucrose solution for approximately two days post fixation. The same protocol, described above, was used for ZENK staining.

Statistical Analysis and ZENK Quantification:

As in Experiment 1, three sections were counted and averaged for both medial and lateral portions of each of the following regions of the nucleus taeniae: 7.50-7.25 region (more anterior); 7.25-7.00 region; and 6.75-6.50 region (more posterior) for each bird. A t-test was done to determine if there were differences in cell counts for these regions. A quadratic discriminant analysis was done on principal component scores of ZENK counts, as described in Experiment 1.

Results:

In both experiments, significant differences in cells positively labeled for ZENK were found between pair bonded birds and non-pair bonded birds in the anterior portions of regions 7.5-7.25 (Experiment 1: $t=2.49$, $p<.05$; Experiment 2: $t=3.2$, $p<.05$) and 7.25-7.00 (Experiment 1: $t=2.70$, $p<.05$; Experiment 2: $t=2.95$, $p<.05$). Sex differences were not seen in pair bonded sample in either group.

Interestingly, ZENK expression in the pair bonded group was not correlated with time spent with mate (Experiment 1:$r(3)=0.127$ $p>.05$; Experiment 2: $(r(8)=0.13$ $p>.05)$. Although most birds had a preference for their mate as determined by the y-shaped preference test, some pair bonded birds did not. In addition, in the pair bonded birds that did have preferences for their mates, percentage of time spent with their mate and amount of time spent with their mate varied greatly (Table 1)

Table 1: Preference test

Dove	Percentage of time spent with mate
1	67%
2	59%
3	63%
4	39%
5	61%

Table 1a: Percentage of time subject spent with pair bonded mate in Experiment 1.

Female	Percent of time spent with mate
1	72%
2	38%
3	100%
4	22%
5	62%
6	89%
7	62%
8	82%
9	54%
10	100%

Table 1b: Percentage of time female subject spent with pair bonded mate in Experiment 2.

The principal component analysis conducted on ZENK cell counts showed that three components (Experiment 1) and four components (Experiment 2) accounted for 95% of the variance in our data. Principal component scores were assigned to the data.

In Experiment 1, birds were classified using both a linear and quadratic discriminant analysis that was based on principal component scores assigned to the data.

The linear discriminant analysis showed a misclassification error rate of 30 percent while the quadratic discriminant analysis was able to classify whether birds were pair bonded or not with 100% accuracy (See Figure 2).

Figure 2: Discriminant Analysis classification

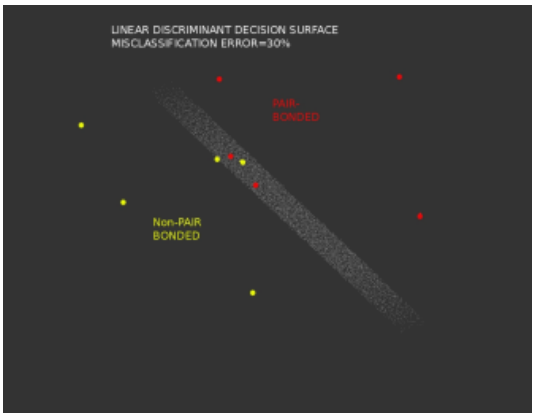


Figure 2a: Classification using a linear discriminant analysis for Experiment 1.

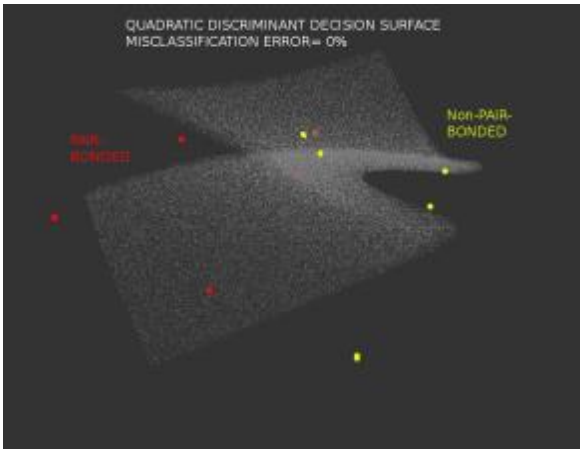


Figure 2b: Classification using a quadratic discriminant analysis for Experiment 1.

In Experiment 2, principal component scores based on cell counts were classified using a quadratic discriminant analysis. The quadratic discriminant analysis was able to classify whether birds were pair bonded or not with 90% accuracy. This is consistent with

preliminary data that classified doves as either pair bonded or not bonded with 100% accuracy.

Discussion:

The results gained from both Experiment 1 and 2, suggest that ZENK expression patterns can be used as a neural marker for pair bonding indicating that this region encodes stimulus properties of the perceptual and motor manifestations associated with pair bonding and illustrating the region's power of predictability. In both experiments, we assessed whether ZENK counts in the anterior medial taeniae were significantly higher in pair bonded doves than in non-pair bonded doves. Additionally, classification using a quadratic discriminate analysis on principal component scores, based on ZENK counts in all regions, ranging more anteriorly to posteriorly, in the taeniae was able to predict pair bonding with high accuracy. ZENK, a neuronal marker that is used to measure changes in behavioral state, is considered to be highly conserved among species (Long and Salbaum 1998). The high predictability of ZENK expression in the nucleus taeniae for pair bonding supports that pair bonding is encoded in the rewards system and suggests that this marker for pair bonding may be retained across multiple species.

The results of our preference tests mimic that of triad tests (Clavijo and Cheng 2007), indicating that doves do not always prefer to spend time with their mates, and collaborate the observation that straying behavior is found amongst pair bonded animals. Inherent in these preference tests are experimental factors, such as housing conditions, time spent away from mate before the commencement of the test, and other procedural factors that could impact the results of the test. Although all birds were separated for a

week pre-preference test, and should have therefore been separate long enough to control for the "coolidge effect" (Dewsbury 1981) , this may still be one of the confounding factors that could account for birds not choosing their mates during preference tests. Because only a small percentage of pair bonded doves did not prefer spending a greater amount of time with their mate, we determined that the Coolidge Effect has little impact on preference results. These and other unknown factors could complicate the interpretation of the preference test and make it difficult to determine whether the results of the preference test are solely determined by pair bonding.

The amygdala, the mammalian counterpart to the nucleus taeniae, has been linked to social and survival behaviors such as fear (Maren et al 1996, Campeau et al 1995) and the processing of emotional memories (Cahill et al 1995, Hamann 2001). The nucleus taeniae has been linked to fear in birds (Brito et al 2011), however, the specific region of the taeniae involved was not specified. This, together with data from analysis of courtship-like behavior in this region (Svec 2009), indicate that separate areas of the nucleus taeniae are involved in mediating pair bonding associated behavior. Interestingly, a lesion study assessing social behavior in ring doves have found that taeniae lesioned females will nest coo (a courtship behavior that mediates the reproductive-endocrine system) at a higher rate than non-lesioned doves, bypassing the natural fear response females normally have towards unfamiliar male doves. (Chen et al 2006). This suggests that the nucleus taeniae can exact an influence on the rate of courtship behavior by controlling fear factors (Cheng et al 1999). Svec et al have shown that courtship-like behaviors, such as preening, are associated with ZENK expression in the taeniae (2009). The taeniae, therefore, is an integrative hub of various factors

contributing to the establishment of pair bonding. Whether the nucleus taeniae functions to aids in a behavioral manifestation of the discrimination of mates from non-mates has not been explored, however, this may be the case and is currently under study.

The taeniae is ideally connected for behaviors associated with reproductive strategies via various distinct regions of the archistriatum, the nucleus accumbens, and other areas responsible for visual and olfactory input (Cheng et al 1999). In follow-up lesion studies, discrete regions of the taeniae as well as regions associated with reproductive and courtship behavior will be lesioned in order to evaluate the connectivity of the taeniae that support the maintenance and formation of pair bonding through its afferent inputs.

Our study has served to highlight the importance of regional versus global analysis when it comes to cell quantification. Previous studies that assessed sexual behavior, fear behavior, courtship behavior, pair bonding-associated behavior, and other forms of social behavior in general (Svec et al 2009; Brito et al 2011; Thompson et all 1998; Cheng et al 1999) have measured the taeniae globally, that is, they analyzed the taeniae as a whole rather than measuring regions of interest in the taeniae that might be implicated in a particular behavior. In this experiment, we used the classifier analysis procedure on ZENK expression cell counts in all regions and did so by discriminating between different regions of the nucleus taeniae. Statistical differences in cell counts, however, were only seen in the most anterior region.

In summary, we have demonstrated an exceptional predictive power of ZENK expression of the nucleus taeniae for pair bonding that appears to override any confounding factors of testing procedures common in behavioral measurement.

Addendum:

In this addendum we address three issues: 1.) whether ZENK counts in the nucleus taeniae from previous experiments (when the data is taken together) are different in bonded and non-bonded doves, 2.) whether differences in ZENK cell counts in these groups are specific to the nucleus taeniae or if they could be seen in another area known to mediate aspects of reproductive behavior, the nucleus accumbens, 3.) whether performance on preference tests during experiments is consistent across multiple trials.

In both experiment 1 and experiment 2, we assessed similar variables, mainly whether ZENK cell counts across multiple regions of the taeniae was different in doves that were pair bonded versus those that were not pair bonded. There was, however, a slight difference between both studies. In the first experiment, the subjects were a small group of mixed sex doves. The second experiment had a larger number of subjects that were of the same gender, females, with a small subgroup of males for comparison. In the following section, we conducted further analysis to determine whether combining these data, while accounting for variance due to experimental conditions and the random variable of running an additional experiment, resulted in consistent differences in amount of positively labeled ZENK cells in bonded and non-bonded groups (1). Although positively labeled ZENK cells were found exclusively in the taeniae region of zebra finch that performed bonding related behaviors (Svec et al 2009) following whole brain analysis of labeled cells, in the following study we conducted further analysis comparing ZENK expression in the nucleus accumbens, a nucleus associated with other mating related behaviors, between bonded and non-bonded doves (2).

As suggested in the above experiment, preference is subject to several experimental factors, including time spent with mate and housing conditions prior to the initiation of the preference test. An additional experiment was run to determine whether preference in doves is retained after bonding (3). Doves were exposed to their mate and a novel dove for multiple trials to determine whether preference varies across trials and whether doves in the non-bonded group differed in the amount of preference retained for an animal during the preference test than doves in the bonded group for their mate.

Experiment 1: Analysis of ZENK cell counts in the nucleus taeniae with data from both experiments

Statistical Analysis:

A generalized linear model was used to determine whether doves if pair bond status affected amounts of positively labeled ZENK cells in the nucleus taeniae in both experiments. A log-link function was used in this analysis because variance between our groups was different. We used number of ZENK cells as a dependent variable and region of interest, sex of the bird, pair bond status, and experiment number as factors in our analysis.

Results:

Sex of the bird being analyzed ($X^2(1, N=125) = 0.013$, $p=0.86$) and which experiment the ZENK labeled cells came from (($X^2(1, N=125)=0.258$. $p=0.611$) did not impact our model. There were, however, differences due to bond status (($X^2(1, N=125)=4.09$, $p=0.043$ and the region of interest (($X^2(4, N=125)=22.02$, $p=.000$) we

analyzed (See figure 1).When assessing parameter estimates for different regions of interest to determine whether they accounted for differences in our model, we found that there were differences in the following regions 7.5 medial (X2(1, N=125)=17.07, B=0.474, SE=0.1148, p=.000), 7.25 medial (X2(1, N=125)=6.92, B=0.301, SE=0.1146, p=0.009) and 7.25 lateral ((X2(1, N=125)=6.246, B=0.286, SE=0.1144, p=0.012). No differences were found in 7.25 lateral ((X2(1, N=125)=0.423, B=0.074; SE=0.1140; p=0.515) and 6.75 (parameter set as zero because it was deemed redundant).

Figure 1: ZENK labeled cells in taeniae

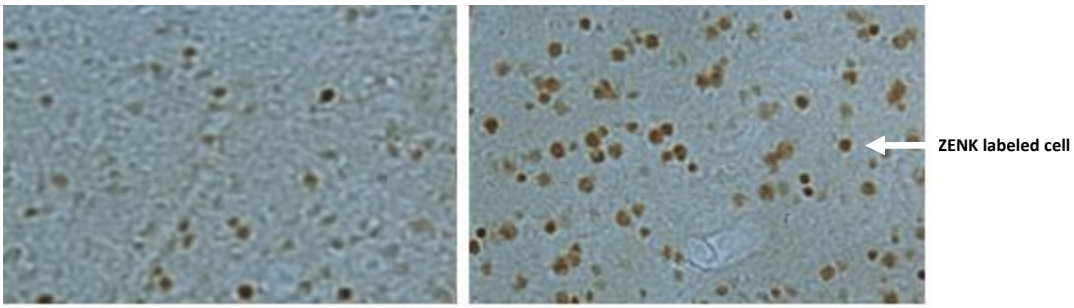


Figure 1: Positively labeled ZENK cells in non-bonded (left) and bonded (right) groups in the nucleus taeniae.

Experiment 2: Analysis of ZENK cell counts in the nucleus accumbens

Statistical Analysis:

A generalized linear model was used to assess ZENK cell counts in the nucleus accumbens (same birds used in previous study) to determine whether the effect of pair bonding on positively labeled ZENK counts was specific to the nucleus taeniae. Three sections were taken from each bird and the average of the labeled ZENK counts in these sections was taken. We used number of ZENK cells in the nucleus accumbens as a

dependent variable and sex of the bird, pair bond status, and experiment number as factors in our analysis.

Results:

Sex of the bird (X2(1,N=26))=3.104, p=0.078) which experiment the ZENK labeled cells came from ((X2(1,N=26))=0.097, p=0.756), and bond status ((X2(1,N=26))=0.780, p=0.377) had no impact on our model (See figure 2).

Figure 2: ZENK labeled cells in nucleus accumbens

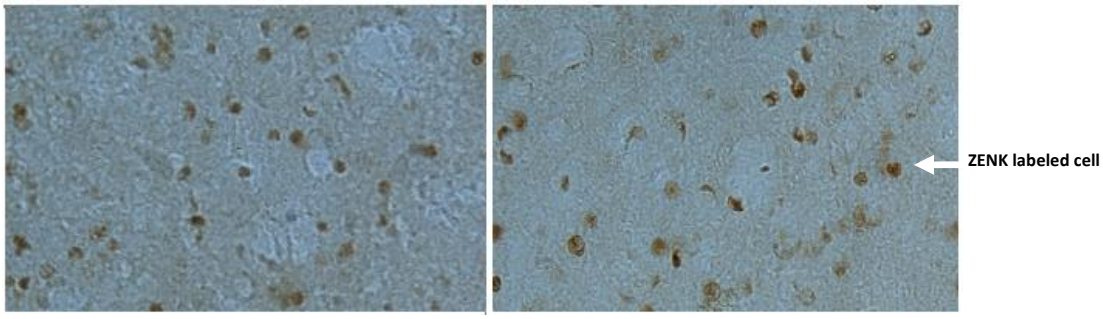


Figure 2: Positively labeled ZENK cell in the nucleus accumbens of non-bonded (left) and bonded (right) doves

Experiment 3: Preference tests after multiple exposures

Methods:

To determine whether choice for a mate was consistent during preference tests, we ran a separate experiment analyzing percent preference towards mate across multiple (three) trials. Ten females were divided into two groups, a non-bonded group (N=5) and a bonded group (N=5). Females in the bonded group were allowed to go through the breeding cycle to establish a bond (Morris and Erickson 1971). Females were separated a

day prior to the initiation of the preference test. Bonded (N=5) and non-bonded (N=5) females were allowed to go through the preference tests, as described in experiment 1, once every other day for the period of six days. A repeated measures general linear model was run assessing percent preference for mate across these three trials. We used percentage of time spent with mate as our dependent variable and bond status and as a between group factor. Trial number was assessed as a within group factor. We ran another repeated measures general linear model to determine whether doves in either group were more likely to spend time in neutral territory across multiple trials.

Results:

Analysis of within group effects found no differences between trials run ($F(1,8)= 1.884$, $p=0.207$). Differences in percent preferences due to bond status, however, were found $F(1,8)=22.112$, $p=0.002$. We assessed whether female doves in the bonded group were more likely to spend time with males than in neutral territories during the preference test than the females in the non-bonded group. There was no difference in percent spent in neutral territory across trials ($F(1,8)=1.227$, $p=0.3$) and bond status ($F(1,8)=0.570$, $p=0.472$) did not impact the amount of time spent in neutral territories.

Further Discussion:

ZENK counts in the nucleus taeniae and nucleus accumbens:

Consistent with our previous results, we found difference in positively labeled ZENK cells between doves that were bonded and those that were not bonded in anterior medial portions of the nucleus taeniae. These differences were not affected by sex of the

bird or the experiment which the bird was run in. When comparing ZENK labeled cells in the nucleus accumbens we found no difference in the amount of ZENK labeled cells in birds that were bonded and not bonded. Similar effects were seen for sex and experiment number. Our results suggest the effects of bond status are seen specifically in the nucleus taeniae. In bird species, immediate early genes have been found to be expressed in the nucleus accumbens after males are exposed to females for a period of 40 minutes (Husband 2004), particularly after sexual behaviors have taken place (Tlemcani et al 2000). Interestingly, doves that had formed a pair bond had undergone the breeding cycle, exposing them sexual behaviors, while those that were in the non-bonded group were not exposed to this. You would expect, therefore, that there should be differences in ZENK labeled cells in the nucleus accumbens in doves that were allowed to go through the breeding cycle (pair bonded group) because they partook in sexual behavior. There may be several reasons why no differences were found in the nucleus accumbens between doves in these two groups. ZENK is expressed an hour following performance of behaviors. Doves in both groups did not engage in these behaviors during this time period because they were exposed to females via a preference test which had barriers that prevented sexual behavior. In quails, immediate early gene, Fos, however, has also been found to be expressed in the nucleus accumbens when males were exposed to females in a sexual context as well as behavior (Tlemcani et al 2000). Both animals were exposed to similar contexts during the time of testing, females viewed through a barrier in a preference chamber, accounting for no differences in ZENK counts in the nucleus accumbens between the two groups.

Consistency of preference test across multiple trials:

Preference in pair bonded doves is retained across multiple trials, but is contingent on bond status, suggesting that doves that formed pair bonds consistently prefer to spend time with their mate. In our original study, some pair bonded doves did not prefer to spend time with their mates during laboratory preference tests. The dependability of preference tests across trials during our follow-up study suggests that there may be individual differences in response to factors within the testing paradigm influencing performance during laboratory preference tests that may affect the outcome of the test. To further confirm where factors associated with the laboratory based test influence preference, another measure of choice, mainly proximity to mate in a more natural setting, should be taken in the same dove. While preference could be a measure of choice for familiar males (a measure of neophobia), rather than inclination towards a mate, analysis of time spent in neutral zones of the preference test versus zones with males found that, while doves in the bonded group were more likely to approach their mate than a novel male, both groups were equally as likely to spend percentage of time in sections of the preference containers with males as compared to neutral zones, indicating that neither group feared approaching females. Additionally, doves perform greater amounts of courtship (nest coo) behavior prior to forming bonds than once they have formed a bond (chapter 2), suggesting that approaching novel animals occurs regularly prior to bond formation. Testing whether preference for a mate is altered when doves have the option of choosing between their mate and familiar females would provide further insight into whether familiarity plays a role in the results from the preference tests.

Chapter 2:

Does the nucleus taeniae mediate pair bonding behavior?

Introduction:

Measuring pair bonding can be complex and is classically done by quantifying reproductive behaviors, assessing proximity to a mate in the group of other animals of the same species, or measuring time spent near a mate via a lab created preference test (Wickler 1975; Morris and Erickson 1971; Aragona et al 2006; Silcox and Evans 1981). While many of these measures are valuable in understanding the emotional attachment necessary for a pair bond, there are factors that may interfere with the results of these tests. Reproductive and courtship behaviors may decline after a period of acclimation, a phenomenon known as the Coolidge effect (Dewsbury 1981), and therefore animals may perform higher amounts of these behaviors towards a novel stimulus after being housed with their mate for an extended period of time. This acclimation process is compatible with what is known about pair bonding's role in survival behaviors, allowing the animal to forgo aspects of courtship behavior in order to be able to more speedily partake in reproductive behaviors (Kleiman 1977; Parker 1974). In addition, factors such as time of day (Lovlie and Pizzari 2007; Ottinger et al 1982; Davies 1974) and time of season (Sharp 1996; Cheng 1979) have been found to influence amount of courtship and reproductive behaviors seen in some species.

The reproductive cycle, in ring doves, involves a measurable set of hormonally governed behaviors that are arranged in a predictable order beginning at bow cooing, a

sexually dimorphic behavior which establishes both the sex and identity of the courting male (Fusani et al 1997) and ending in the rearing of a squab (Miller and Miller 1958; Lovari and Hutchison 1975; Lehrman 1964). The reproductive cycle will recommence after a squab reaches maturity and can provision for itself or can recommence if a male is introduced to a novel female and has not been exposed to sufficient stimulus in order to continue in incubation and squab rearing (Friedman and Lehrman 1968; Cheng 1979). The nest coo, a behavior that, in the case of the female, is integral in activating hormonal processes required for self-stimulation in female ring doves leading to follicular maturity leading to ovulation (Cheng et al 1998), is speculated, in the case of the male, in functioning in attracting females to the nest site (Davies 1974). The nest coo, as well as other behaviors such as preening and wing flipping, are used as measures of reproductive behavior in doves (Miller and Miller 1958). A pair that goes through a reproductive cycle prefers to be in proximity to their each other and will prefer to spend time with the other when placed in a preference test (Morris and Erickson 1971), suggesting that they have an attachment to their mate. Preference tests in our lab confirm this (Clavijo and Cheng 2007). Although fairly consistent, these preference tests do not always produce expected results. Not all doves that go through the reproductive cycle prefer spending time with their mates. A recent study (chapter 1), has suggested that ZENK activated cells in the nucleus taeniae serve as a marker for pair bonding in doves and may be more accurate than preference tests alone. Together both ZENK activated cells in the nucleus taeniae and partner preference can be used as tools for measuring pair bonding once a pair bond is formed.

While our previous study provides evidence that the nucleus taeniae encodes a pair bond, whether this region is responsible for regulating bonding behavior is unknown. Tract tracing in ring doves and European starling have shown a large fiber bundle terminating in hypothalamus, known as the hypothalmatic-occipitomescenphalic tract, as well as sensory input, from the olfactory bulb and other sensory regions, suggesting that the nucleus taeniae is homologous to the mammalian amygdala (Cheng 1999; Reiner 2004). Functional studies collaborate this finding. The nucleus taeniae has been found to be involved in fear and sexual conditioning (Brito et al 2011; Burns-Cusato et al), perception of emotional stimuli (Marzluff et al 2012). A lesion study can establish a causal relationship between the nucleus taeniae and pair bonding. If damaging the taeniae destroys bonding, and the dove fails to make a choice towards the mate, then the taeniae likely mediates the manifestation of this behavior. If, however, the dove is able to choose its mate from another animal when the taeniae is damaged, then the taeniae would be simply encoding preference for a pair bonded mate (responsive to a mate).

The nucleus taeniae has been found to mediate emotional behaviors, moderating activities that may be involved with the disruption of these processes. Cheng et al found that lesions to the nucleus taeniae in ring doves increased nest coo behavior, a phenomenon that was attributed to a diminishing of fear, which is mediated by the nucleus taeniae, in lesioned doves (1999). Consistent with these findings, Absil et al found that quails showed a disinhibition in courtship behavior, but found no change in copulatory behavior after taeniae lesions, suggesting that this area may function in regulating these behaviors via suppression of behaviors mediated by other regions (2002).

While other findings have not substantiated these effects (Thompson et al 1998), discrepancies have been attributed to differences in lesion sites (Absil 2002).

If the taeniae functions in encoding bonding behavior, as suggested in our previous study (Chapter 1), it may also function in modifying behaviors, particularly reproductive and courtship behaviors. Anatomical data suggests that the taeniae is well connected for moderating reproductive behavior, in particular nest coo behavior (Cheng et al 1999). Whether forming a pair bond alters reproductive or courtship behaviors towards a mate, as suggested above, can be tested. In order to understand the role of bonding, and have the appropriate context to interpret taeniae lesion studies, this question will be addressed in this study.

The present study seeks to establish the role of the nucleus taeniae and its functions in mediating pair bonding and bonding associated behaviors in ring doves. In experiment 1, preference for a mate will be assessed both preceding bilateral taeniae lesions and after lesions in doves to determine if the taeniae has a role in preference for a bonded mate, a measurement of proximity which is indicative of the emotional tie needed for pair bonding. Pair bonding has been speculated to alter behaviors of the breeding cycle. Once a dove goes through a breeding cycle, and therefore is pair bonded (Morris and Erickson 1971), a dove will exhibit less courtship behavior during the following breeding cycle (Erickson 1973). In order to determine whether bond status affects courtship behavior, we tested if there were differences in amount of courtship (nest coo) behavior in bonded versus non-bonded doves (experiment 2). We further assessed if courtship behavior changes following bilateral taeniae lesions to determine whether

damage to this area affects bond status and changes in courtship that may result from it (experiment 3).

In previous studies (chapter 1), we found that bond status had an effect on ZENK expression in the anterior medial region of the nucleus taeniae. In these studies, doves were allowed to go through a breeding cycle, exposing them to a number of reproductive behaviors including courtship (nest coo) behavior and differences in ZENK expression were assessed following a preference test, which exposed doves to varying amounts of courtship behavior. In experiments 2 and 3, we test whether courtship behavior has an effect on bond status. In experiment 4, we will evaluate whether courtship behavior is needed to elicit the differences in ZENK expression found in doves of varying bonding statuses (experiment 4), allowing us to determine whether the presence of courtship behavior is necessary for the representation of bonding in the brain.

Methods:

Experiment 1: Does a taeniae lesion destroy a dove's preference for its mate?

Subjects:

Male ring doves were allowed to complete a breeding cycle, including the rearing of a squab and were housed in an AAALAC accredited facility at Rutgers University, Newark. Doves were put through a y-shaped triadic preference test created by our laboratory (Clavijo and Cheng 2007) to insure all doves used for this experiment had an initial preference for their mate. Doves that did not show a preference were discarded. Twelve doves were selected to participate in this experiment. Doves were then randomly

divided into two equal groups (a taeniae lesion group and a sham lesion group) and were housed with mates for at least one week prior to the lesion surgery.

Electrolytic lesion surgery:

Surgeries were done in accordance with protocols approved by AAALAC at Rutgers University, Newark. Doves were anesthetized using Choropent (2.5 ml/kg) approximately 30-40 minutes before surgery. Doves were checked prior to surgery to insure they were non-responsive to an inter-digit (toe) pinch along with other measures to insure they were asleep and were given an additional dose of Marcaine (.0125%) which was administered subcutaneously along the incision site prior to the lesion. The dove's head was fixed on a stereotaxic instrument at a forty-five degree angle. An incision was made along the midline of the skull and electrodes (0.03 mm) were inserted into the following coordinates: 4.5 mm anterior (A), 4.5 lateral, and 5.5(L), and 5.5mm ventral (V), in accordance with previous lesion studies done in our laboratory (Cheng 1992). One milliamps of positive current was administered for 30 seconds for each of the bilateral lesions. Doves that were in the sham lesion group underwent a similar surgical procedure, but no current was administered during surgery for subjects in this group. After the surgery, doves were sutured and allowed to recover in isolation for a week before the initiation of behavior and preference testing.

Behavior Testing:

Lesioned and sham-lesioned males were placed in a cage with the female they were bonded to for an hour testing block. A trained observer that was unfamiliar with the

purpose of the study recorded behaviors exhibited by the male and his female partner during this time, specifically the number of reproductive and courtship behaviors including bow coo, nest coo, cackling, wing flipping, preening, billing, mounting attempts, and copulation. After the termination of each behavioral observation, the males were separated from their mate for a week's period of time in preparation for the preference test.

Triadic y-shaped preference tests:

Doves were placed in a triadic preference test created by our laboratory (see Chapter 1, figure 1). A series of steps took place before the initiation of the test in order to insure that the doves were acclimated to the test chamber. Doves were allowed to wander the preference test apparatus for 15 minutes (without any dividers in place) in order to become familiar with the chamber. Dividers were then placed in the preference apparatus and the male subject was placed in a chamber that gave it direct visual access to two females, its mate and a novel female with whom the male has had no experience, but is not allowed access near their chambers. The male was held in his chamber in this state for 15 minutes. The initiation of the preference test occurred when the divider preventing the male's access to the females' chambers was lifted and the male was allowed to explore the chamber for 1 hour. The side the female mate was placed in was switched every other trial in order to prevent side preference during the test. Preference was measured by the percentage of time spent in the proximity of either female's side chambers in relation to the other. Males that spend more than 60% of the time with their mate, an above chance value, are considered to have preference for them. Preference tests

were done before the bilateral taeniae lesion in order to insure that males were attached to the females they were bonded to before the onset of the study and were done after the lesion study in order to determine whether this preference was maintained post-lesion.

Brain extraction and preparation:

Doves were overdosed with chloropent and sacrificed an hour after the termination of the preference test. Experimental doves were perfused using a 4% paraformaldehyde solution. Brains were extracted and fixed in 4% paraformaldehyde solution overnight. Tissue was stored in a 30% sucrose solution for approximately two days post fixation. Brains were frozen and cut into 30 micron coronal sections. Sections were stained with cresyl violet and checked for lesion site accuracy under the microscope.

Results:

Males that were sham lesioned spent significantly higher percentage of time with their mate ($t(10)=4.606$; $p=0.0005$) during preference tests (See Figure 3). On average they spent 88.5% (SD=11.27) of their time with mates while those that were sham lesioned spent 50.1% (SD=16.99) of their time with mates. Males that were lesioned (M=150.7; SD=73.25) had higher rates of nest coo ($t(10)=3.1$; $p=0.0058$) than those that were not lesioned (M=53.83; SD=18.52) while bow coo behavior was similar ($t(10)=0.7$; $p=0.25$) for both the sham lesioned (M=46.33; SD= 8.524) and taeniae lesioned groups (M=50.83;SD=12.98) (See figure 4). Other behaviors were performed at such low rates that statistical analysis was not possible.

Figure 1: Cumulative lesion site area

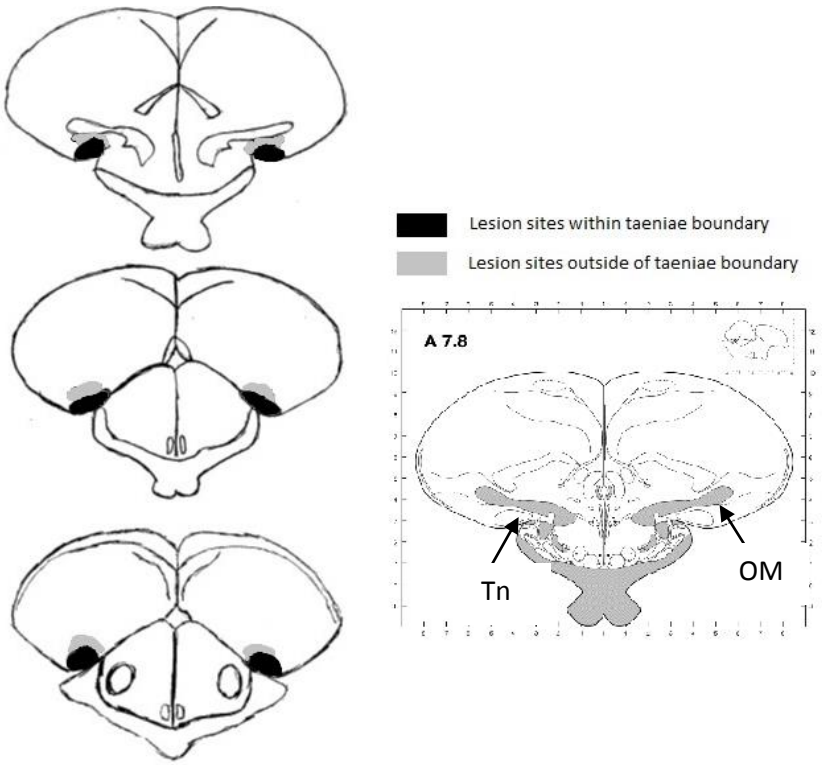


Figure 2: Image of lesion site (4x objective)

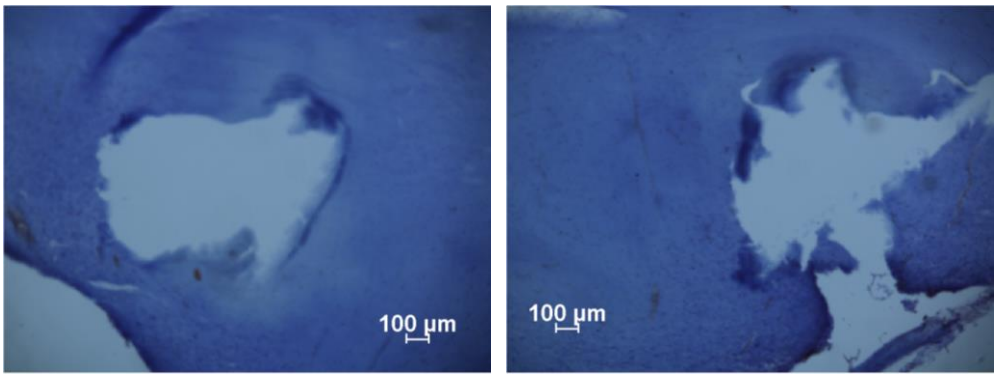


Figure 3: Image of lesion sight for left (as seen on left) and right (as seen on right) lesion sites for one bird.

Figure 3: Percentage of time spent with mate

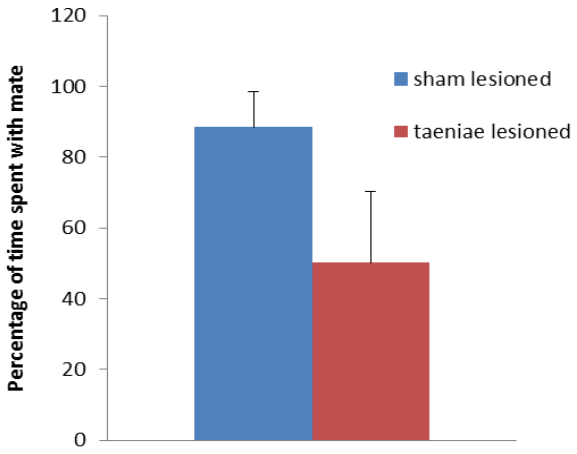


Figure 3: Percentage of time spent with mate in taeniae lesioned and sham lesioned group during preference test

Figure 4: Amount of bow and nest coo behavior

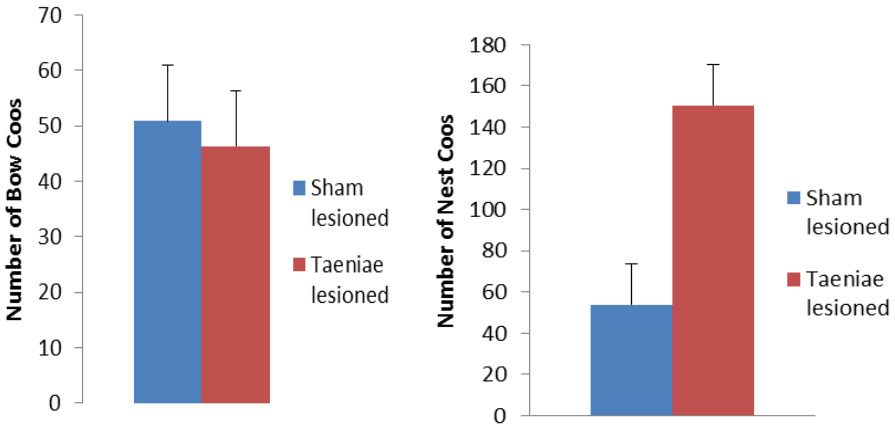


Figure 4: Amount of bow coo behavior (greeting behavior) and nest coo behavior (courtship behavior) performed by males in the taeniae lesioned and sham lesioned groups

Experiment 2: Do bonded male doves court (nest coo) less towards their mates than non-bonded doves do towards a stimulus female?

In the previous experiment, amount of nest coo was increased following taeniae lesions in bonded ring doves. To determine whether this behavior resulted from the elimination of bond status, we first have to assess if there are differences between bonded and non-bonded doves in the intensity of courtship behavior performed. The following study assesses whether doves that are bonded naturally perform different amounts of courtship behavior than doves that are not bonded.

Methods:

Subjects:

Eighteen male doves raised in an AAALAC accredited facility in Rutgers University, Newark were dived into three groups. The bonded group (n=6) was allowed to go through a breeding cycle, including the rearing of a squab, the non-bonded group was never allowed to bond and was housed in isolation (n=6), and a non-bonded group that was housed with a novel female that was rotated out every two days (n=6) in order to prevent the formation of pair bond. A day before the initiation of behavior tests, males were removed from their typical housing conditions and were housed in isolation.

Behavior tests:

As in experiment 1, males were put into a cage with their mate (if in bonded group) or into a cage with a novel female (if in non-bonded group) for a period of an hour. Behaviors performed were recorded by a trained observer, which was instructed to record the number of nest coo (courtship) behaviors performed by males in each group.

Results:

There was a significant difference between all three groups in the number of nest coos produced (F(2,15)=13.74; p=0.000407) (See Figure 5). Doves in the non-bonded group that were previously housed with females which were switched ever two days (M=297.7;SD=75.47) had a significantly higher amount of nest coo (Mdiff=155.8; p=0.0093) than those in the bonded group (M=141.8; SD=29.53) and a significantly higher amount of nest coos (MDiff=232; p=0.0003) than doves in the non-bonded group that were housed in isolation (M=65.67; SD=108) prior to the experiment. Doves in the non-bonded group whose members were housed in isolation prior to the experiment did not significantly differ to pair bonded doves in the amount of nest coo behavior performed (MDiff=76.2; p=0.2418).

Figure 5: Amount of nest coo behavior

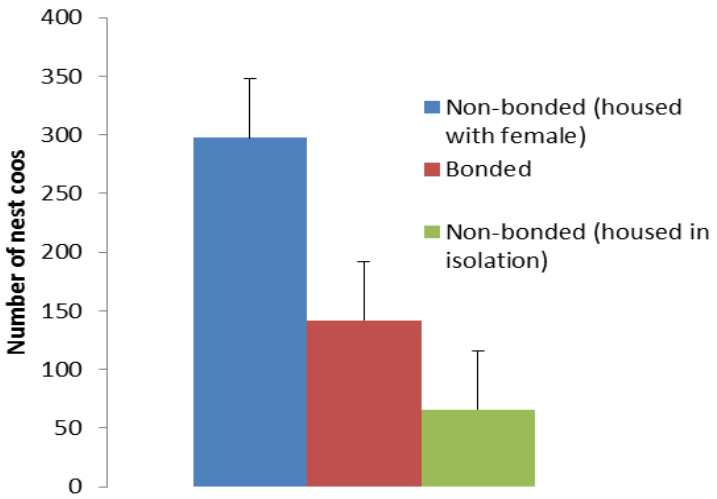


Figure 5: Amount of nest coo behavior performed by non-bonded, bonded, and non-bonded doves in isolation prior to lesion

Experiment 3: Do taeniae lesions affect intensity of nest coo (courtship) behavior due to bond status?

Experiment 1 showed that taeniae lesions increase the intensity of nest coo behavior. We found that pair bonded doves naturally produce less nest coo behavior towards their mates than doves that are not bonded towards a stimulus female (experiment 2). If bond status is eliminated by taeniae lesions, we suspect that reproductive behaviors, such as courtship (nest coo) behavior, that are moderated by pair bonding will be affected by taeniae lesions. In order to confirm these results, the following study tests whether doves that are bonded and not bonded differ in the amount of courtship (nest coo) behavior following taeniae lesions.

Methods:

Subjects:

Ten male doves raised in AAALAC accredited facilities in Rutgers University, Newark were equally dived into two groups: a bonded group (n=5) and a non-bonded group (n=5). The bonded group was allowed to go through a complete breeding cycle including the rearing of a squab. All males underwent lesion surgery in accordance to procedures listed in experiment 1. Doves were housed in isolation after surgery and allowed to recover for one week prior to the initiation of behavior tests.

Behavior tests:

Males were introduced into a cage with either a female they were bonded to (bonded group) or a novel female (non-bonded group). A trained observer recorded amount of

courtship and reproductive behavior during an hour test period. In particular, nest coo behavior was assessed as it is speculated to be related to courting the female and attracting her to the nest site.

Lesion site confirmation:

Doves were sacrificed via chloropent overdose an hour after the termination of the behavior tests and their brain was extracted and checked for lesion accuracy, in accordance to procedures used in experiment 1 (see figure 6 and 7).

Figure 6: Cumulative lesion site area

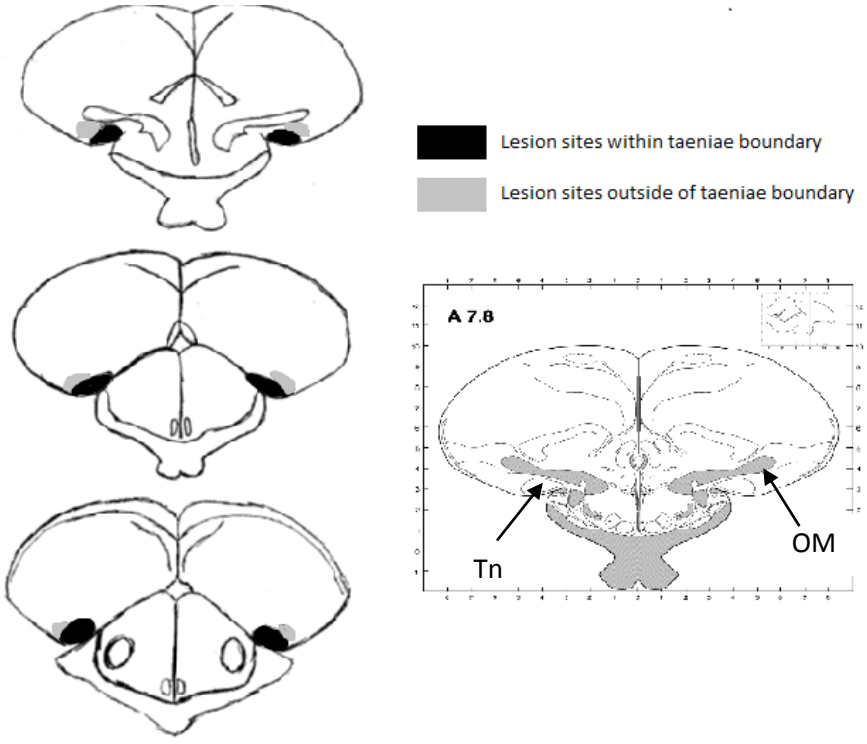


Figure 7: Image of lesion site

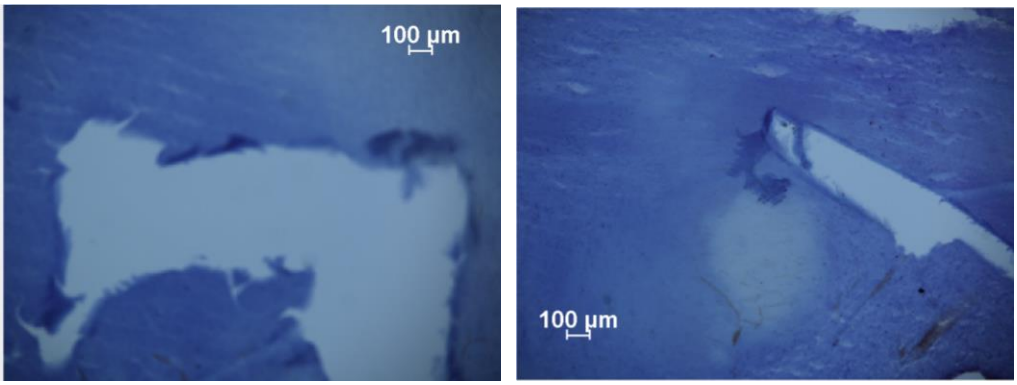


Figure 7: Image of lesion site (4x objective) for left (as seen on left) and right (as seen on right) lesion sites for one bird.

Results:

Differences in nest coo behavior between doves of different bond status after the taeniae was lesioned were assessed. Amount of nest coo behavior during behavior tests in doves that were bonded (M=168.2; SD=16.48) and non-bonded (M=184.6; SD=24.46) was not significantly different in these two groups (t (8)=1.2; p=0.13) after lesion (see figure 8).

Figure 8: Amount of Nest coo behavior

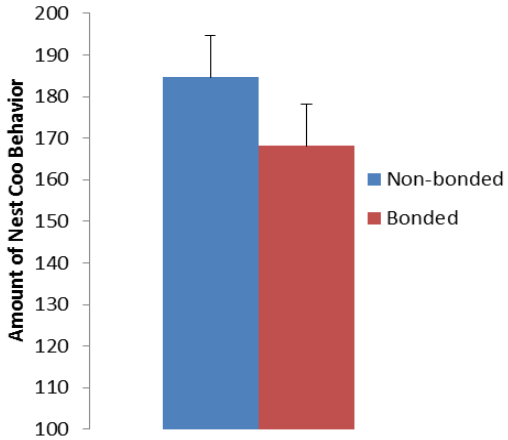


Figure 8: Amount of nest coo behavior performed by males in non-bonded and bonded group following bilateral taeniae lesions

Experiment 4: Is a brief exposure (no courtship behavior) sufficient to produce differences in ZENK expression in doves that are bonded versus those that are not bonded?

Results from experiments 1-3 suggest that bond status influences amount of courtship behavior produced in doves. In chapter 1, we found that there is a greater amounts of ZENK expression in bonded doves than in non-bonded doves. This suggests that amount of courtship behavior may be relevant for activation of ZENK expression. The following experiment assesses whether the brain can activate ZENK expression when exposed to a pair bonded mate in the absence of courtship behavior in doves that have previously gone through a breeding cycle.

Methods:

Subjects:

Ten females were divided into two groups, a non-bonded group (N=5) and a bonded group (N=5). Females in the bonded group were allowed to go through the breeding cycle to establish a bond (Morris and Erickson 1971). Females were separated from their mate a day prior to the initiation of experimental exposure. Females were put into a cage and exposed to their mate (if in bonded group) or a novel male (if in non-bonded group) for a period of 10 minutes.

ZENK immunostaining:

After the 10 minute brief exposure period, females were separated from males for one hour and then sacrificed via a lethal injection of Chloropent. Females were perfused intracardially with 0.9% saline followed by 4% paraformaldehyde in 0.1 M phosphate buffered saline. The brains were stored in a 25% sucrose solution overnight. Brains were frozen and coronally sectioned (30 um). Sections were washed and stained in accordance to procedures described by Svec et al (2009). Briefly, sections were rinsed in PBS and incubated in a 0.5% hydrogen peroxide solution for 15 minutes. After being rinsed in PBS, sections were blocked with a 5% normal donkey serum solution. Following an hour period of incubation, sections were incubated in primary antibody anti-Egr-1 (1:2000) overnight. Tissue was washed and incubated in a dilution (1:500) of secondary Biotin-sp-conjugated donkey anti-rabbit IgG antibody. After being incubated for an hour, tissue was washed and processed with a Vectastain ABC Elite Kit (Vector laboratories). Diaminobenzidine (DAB) in a 0.0075% hydrogen peroxide was used to for visualization. Tissue was then mounted onto slides and coverslipped. Control sections without primary antibody also underwent this process.

Quantification of ZENK labeled cells:

Positively labeled ZENK cell counts were assessed in the anterior medial region (7.5-7.25) of the nucleus taeniae corresponding to the pigeon brain atlas (Karten and Hodos 1967), where differences in cell counts between bonded and non-bonded birds had been previously found (Dios et al 2013). Three sections from this region were taken from every bird and averaged to produce an average number of positively labeled ZENK counts for each bird.

Results:

There was a significant difference between positively labeled ZENK cell counts in pair bonded (M=15.93, SD=7.85) and not bonded (M=26.16, SD=7.31) doves that were exposed to their mate for a brief (10 minute) period of time ($t(8)=2.132$, $p=0.033$).

Discussion:

Our current study demonstrates that lesions to the nucleus taeniae destroyed preference for a mate suggesting a causal relationship between this region and choice for a mate during a preference test. In the case of ring doves, males that go through a breeding cycle and are afterwards separated for a period of several months will find each other in an enclosure of other doves and will prefer to spend time with each other (Morris and Erickson 1971) despite a period of dissociation. Males that have reached the end of a breeding cycle prefer to spend time with their mates during laboratory run preference tests (Morris and Erickson 1971). Laboratory preference tests measure the amount of time in proximity to a mate while controlling for effects of choice due to location, namely they are a measure of attachment (Wickler 1975). While bilaterally lesioning the nucleus taeniae damages preference for mates (experiment 1), it does not destroy courtship behavior (nest coo), suggesting that doves are able to court without attachment. Courtship behavior increases following taeniae lesions in ring doves (experiment 1). Doves that are pair bonded (experiment 2) produce nest coo behavior at a lower intensity than those that are not bonded prior to lesions. This effect was erased when doves that were bonded were housed in isolation prior to behavior tests, suggesting that experience plays a role in the performance of courtship behavior. Consistent with previous studies

done in our laboratory (Cheng et al 1999), doves that underwent taeniae lesions performed significantly more courtship behavior than those that were in the sham lesion group, suggesting that taeniae lesions may impact behaviors that are influenced by bond formation, such as courtship and reproductive behaviors directed towards the bonded mate. It is not clear if this is a direct effect or if it is moderated by other taeniae functions since the whole taeniae was lesioned.

Bond status did not affect the number of nest coos performed by birds following bilateral taeniae lesions (experiment 3), confirming findings in experiment 1 which suggested that taeniae lesions impact courtship and reproductive behaviors, which are moderated following the formation of a pair bond. Once bonded, doves attenuate the amount of nest coo behavior performed and direct it towards their mate (Erickson 1973). While lesions to the nucleus taeniae influence the intensity of courtship behavior (nest coo) via the destruction of the bond, once the pair has gone through the breeding cycle, courtship behavior is not necessary for higher levels of ZENK expression in bonded doves, nor is it contingent on the dove being exposed to a choice paradigm. Doves that were bonded and exposed to their mate for 10 minutes, limiting amounts of courtship behavior to negligible levels, showed more amounts of ZENK labeled cells in the nucleus taeniae than those that were not bonded and were shown a novel female. This suggests that the nucleus taeniae functions in mediating a dove's ability to recognize its pair bonded mate. These results are consistent with other findings that suggest that the amygdala functions in the processing of positive emotional memories (Bradley et al 1992; Hamann 2002; Hamann et al 1999). ZENK expression has been found to be higher in bonded doves in the anterior medial region of the nucleus taeniae (chapter 1),

indicating that this area may be responsible for mediating pair bonding behavior. Our lesion studies, however, did not target this region specifically, but instead damaged the whole taeniae (including this area), allowing for other factors to contribute to these results. Further analysis targeting the anterior medial region specifically will confirm that this area is mediating pair bonding.

Together these findings indicate that the taeniae is mediating preference for a mate and moderating the output of courtship behavior (nest coo) that is resulting from bonding. Lesions to the nucleus taeniae in non-bonded quail did not affect proximity behavior or other pre-sexual preparatory behavior (Absil et al 2002), consistent with the idea that changes in nest coo behavior following taeniae lesions are likely attributed to loss of bond status rather than the taeniae's direct mediation of these behaviors. The production of courtship behavior in ring doves is mediated by the ventromedial nucleus of the hypothalamus (VMN). Neurons in the ventromedial nucleus of the hypothalamus (VMN) selectively respond to nest coo (courtship) behavior allowing for the increase of LH which is necessary for ovulation in female doves (Cheng et al 1998). Damage to the VMN blocks doves from performing courtship displays, showing a causal link between this region and nest coo behavior (Bernstein et al 1993; Cheng 2004). The nucleus taeniae is connected to the hypothalamus via its large fiber connection, hypothalmatic-occipitomescenphalic tract (Cheng 1999). This suggests a potential route for the moderation of courtship behaviors, particularly nest coo behavior, towards a mate following the formation of a pair bond.

While pair bond status has been found to moderate the intensity of nest coo behavior, the role of courtship behavior in the formation of a pair bond is not well understood. Pair bonding in ring doves occurs following a breeding cycle (Morris and Erickson 1971), however, whether any behaviors during the breeding cycle, including courtship (nest coo) behavior, function in initiating the process of pair bonding must be assessed to fully understand the role of courtship behaviors in pair bonding.

Chapter 3

Breeding Cycle and its relationship to pair bonding in ring doves

Introduction:

A pair bond is an emotional relationship that can alter the valance of the object of the bond and lead to changes in behavior of the animal experiencing the effects of attachment, a feature central to the formation of a bond. In ring doves, bonding leads to a reduction in courtship (nest coo) behavior (chapter 2) and the directing of courtship behavior towards the animal's mate (Erickson 1973). Lesioning the nucleus taeniae (avian amygdala), an area associated with the maintenance of preference for a mate in ring doves (chapter 2), erases the effects of bonding on courtship behavior and removing the dove's ability to bypass portions of the courtship routine during the breeding cycle. Morris and Erickson found that doves that underwent a breeding cycle, which includes the production of courtship behavior among other reproductive behaviors, recognized the female they went through the breeding cycle with after a seven month separation period and preferred spending time near her in an enclosure of other doves (1971). This is replicated in laboratory run preference tests where the male retained a preference for female (Clavijo and Cheng 2007). These findings indicate that bond formation occurs after the completing of a breeding cycle, however, whether the doves have to go through the entirety of the breeding cycle or whether bonding before then is unknown.

The breeding cycle in ring doves follows a measurable, sequential pattern of behavior (Wallace 1908; Miller and Miller 1958, Lovori and Hutchinson 1975) allowing

for landmark stages during the cycle to be assessed. The breeding cycle is initiated when males engage in a greeting behavior termed the bow coo. Differences in components of bow coo produced by individual males suggests that bow coo may be used to impart information about the male's identity (Fusani et al 1997). After a period of performing this behavior, males will begin to integrate a courtship behavior, the nest coo, into their repertoire, eventually drawing females to the nest site and engaging females in participating, resulting in a duet of nest coos between the male and the female (Erickson 1971; Miller and Miller 1958; Lovari and Hutchinson 1975). The female's own nest coo primes her for participating in later stages of the breeding cycle. Electrophysiological recordings from cells in the ventromedial nucleus of the hypothalamus (VMN), that receives projections from the nucleus ovodalis (avian auditory thalamus), show that stimulation of this region by the female's own nest coo leads to increases in luteinizing hormone (LH) that are necessary for ovulation (Cheng et al 1998). Once the females are primed into producing nest coo behavior, male nest coo behavior decreases and ceases. Female nest coo behavior peaks following the cessation of male nest coo behavior and while continue onto later stages of the breeding cycle, but will eventually also cease in preparation for offspring rearing (Cheng 1979). Females will lay eggs, usually two, (Lehrman 1964; Lehrman 1959) and once the eggs hatch both doves will partake in parental care until their offspring are able to feed themselves (Friedman and Lehrman 1968; Cheng 1979). The breeding cycle recommences following offspring rearing.

Our current study seeks to determine whether going through a full breeding cycle is necessary for the formation of a pair bond in ring doves. We will determine whether

formation of a bond occurs at different landmarks during the breeding cycle that are distinguishable by their prominent behavior pattern, mainly, once they have reached the bow coo stage, nest coo stage, egg laying stage, and the squab rearing stage. In addition to the four previously mentioned landmarks, two additional groups, a group that has gone through the complete breeding cycle and a group that has not gone through any portion of the breeding cycle will be assessed. Cells of bonded doves have been shown to contain higher amounts of cells labeled with a ZENK marker in the nucleus taeniae than those of non-bonded doves (chapter 1). We will compare positively labeled ZENK cells in doves at different phases with doves that are not bonded to assess whether behaviors associated with these phases triggers pair bond formation.

Methods:

Subjects:

Twenty-five pairs of doves and ten single doves that were bred and housed in an AAALAC accredited animal care facility at Newark, Rutgers University were equally divided into six groups. The first two groups consisted of males (n=5) and females (n=5) that did not go through any part of a courtship cycle and were not bonded. The remaining doves were paired and the pairs were split into groups at several landmark periods during the breeding cycle including a bow coo (n=5 male/female pairs), nest coo (n=5 male/female pairs), egg laying (n=5 male/female pairs), squab rearing (n=5 male/female pairs), and full cycle (n=5 male/female pairs.

Behavioral monitoring:

Naïve doves that had not undergone a breeding cycle were paired together in (28x30x17in) cages in an AAALAC accredited animal care facility at Rutgers University, Newark. Doves were monitored by a trained observer for 10 minutes daily until behaviors of interest were observed. The behaviors of interest included those related to different stages of the breeding cycle and included bow coo behavior, nest coo behavior, egg laying, squab rearing, and full cycle. For reference (Lehrman 1964; Lehrman 1959), bow coo will generally occur after the first day of pairing and nest coo behavior will occur after the second day of pairing. The female will lay her first egg between 1 week to 11 days after pairing and her second egg about a ½ a day to a day later. Eggs typically hatch about two weeks after the initial pairing and young are fed until they are 15-25 days old. Once these behaviors were observed, doves were removed from their home cage and into an isolation cage (28x30x17in) were they remained for an hour.

ZENK immunostaining:

After an hour in the isolation cage, birds were sacrificed by a lethal injection of Chloropent and perfused intracardially with 0.9% saline followed by 4% paraformaldehyde in 0.1 M phosphate buffered saline. After being fixed overnight, the brains were stored in a solution of 25% sucrose in 0.1 MPBS overnight. Brains were frozen using powdered dry ice and tissue was cut into 30 micron coronal sections, washed, and stained in accordance to procedures described by Svec et al (2009). Tissue was washed twice with PBS for 5 minutes and incubated in a 0.5% hydrogen peroxide solution for 15 minutes. The tissue was rinsed 3 times in PBS for 5 minutes, blocked with a 5% normal donkey serum solution for one hour, and incubated in primary antibody

anti-Egr-1 (1:2000) overnight. Samples were then washed twice in PBS for 10 minutes and incubated in a dilution (1:500) of secondary Biotin-sp-conjugated donkey anti-rabbit IgG antibody for 1 hour. After incubation, tissues were washed twice in PBS for 10 minutes, processed with a Vectastain ABC Elite Kit in accordance to procedures given by Vector Laboratories, and visualized using diaminobenzidine with a 0.0075% hydrogen peroxide. Tissue was then mounted, dehydrated, and coverslipped. Control sections without primary antibody also underwent this process.

Quantification of ZENK labeled cells and statistical analysis:

Our previous study (chapter 1) found differences in amount of positively labeled ZENK cells in anterior medial portions of the 7.5-7.0 A regions (corresponding to the pigeon brain atlas Karten and Hodos 1967) in doves that were pair bonded versus those that were not bonded. For each bird, positively labeled ZENK cells from three sections in this region was counted and averaged, as done in chapter 1.

We ran a general linear model to assess whether there were differences in ZENK counts due to stage of bonding in males and females. This model has been previously used for similar temporal analyses (Anderson et al 2009; Grim et al 2009). In our analyses, we transformed our data using $\ln(x+1)$ in order to account for variance between groups while allowing for the maintenance of "0" value data (Fletcher et al 2005), an important feature in cell count data where zeros have a meaningful value. For the male group, we included stage of bonding as a factor and number of ZENK counts in male subjects as a dependent variable. The same analysis was run on the female group; however, the dependent variable was the number of cell counts in the female group. The "not-bonded" group was included as a type of stage in our analysis.

Results:

Differences were found between male doves that were not bonded and male doves that had gone through the nest coo phase ($M_{Diff=}$-0.797, SE=0.356, p=0.035), the squab rearing phase ($M_{Diff=}$-0.821, SE=0.356, p=0.030), and the full cycle ($M_{Diff=}$-1.003, SE=0.356, p=0.010). Marginal differences were found between non-bonded doves and doves that had been exposed to females laying eggs ($M_{Diff=}$-0.671, SE=0.356, p=0.072). No differences were found between doves that had not been bonded and those that had gone through the bow coo stage ($M_{Diff=}$-0.487, SE=0.356, p=0.184) (See figure 1).

Figure 1: Number of ZENK labeled cells in males

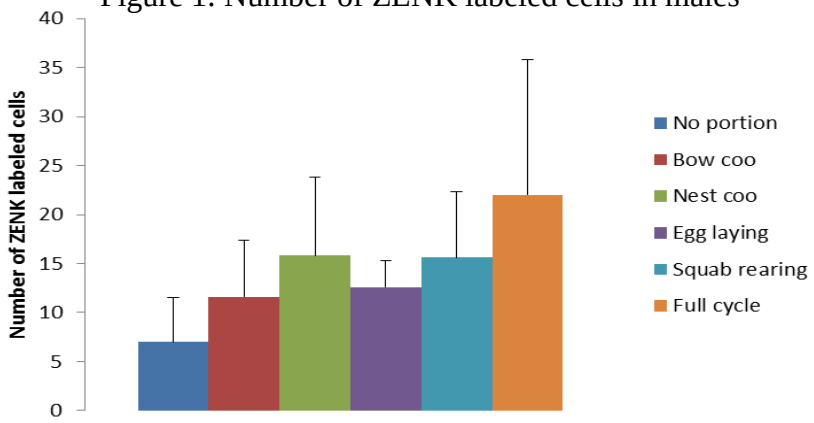


Figure 1: Amount of positively labeled ZENK cells in the nucleus taeniae in groups of ɨd and male doves across different stages of the breeding cycle.

females that had gone through the nest coo phase ($M_{Diff=}$-1.064, SE=0.361, p=0.007) the egg laying phase ($M_{Diff=}$-1.032, SE=0.361, p=0 .009), the squab rearing phase ($M_{Diff=}$-1.254, SE=0.361., p=0. 002), and a full cycle ($M_{Diff=}$-1.469., SE=0.361, p=0.000). No differences were found between female doves that had not been bonded and females that were not exposed to male's bow cooing ($M_{Diff=}$-0-.369, SE=0361, p=0.317) (See figure 2).

Figure 2: Number of ZENK labeled cells in females

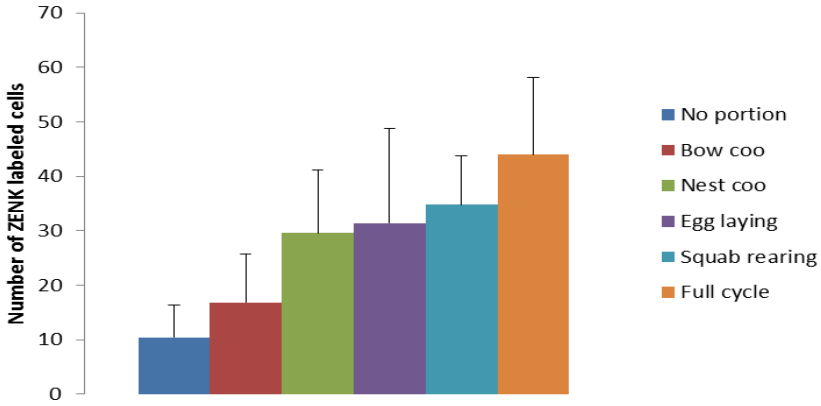


Figure 2: Amount of positively labeled ZENK cells in the nucleus taeniae in groups of female doves across different stages of the breeding cycle.

Discussion:

Doves that have gone through a breeding cycle, and are separated from their mate for several months are able to recognize their mate amongst a group of other doves, prefer to spend time in proximity to them, and prefer to spend time with their mate during laboratory preference tests (Clavijo 2007, Morris and Erickson 1971). Prior to the current study, it was not known at which point during the breeding cycle the formation of pair bonding occurred. We found that both male and female doves had significantly more positively labeled ZENK cells in the anterior portion of the nucleus taeniae when they underwent the courtship (nest coo) portion of the breeding cycle, as well as stages following nest coo.

Our results show that the completion of a breeding cycle is not necessary for the formation of a pair bond, as Morris and Erickson suggested. The findings that nest coo phase is sufficient in forming a pair bond is consistent with the widely understood

evolutionary function of pair bonding-successful breeding. In ring doves, caring for offspring requires a great deal of investment from both males and females. During the period after the eggs are hatched, both parents share in incubating their eggs, and once the squab hatch both doves share in feeding the hatchlings until they are old enough to fend for themselves (Lehrman 1959; Lehrman 1964; Cheng 1979). The formation of a bond prior to breeding ensures that the pair is more likely to invest in offspring rearing. In doves, the stimulation of reproductive-endocrine changes in the female leading to ovulation requires that the female hears her own nest coo, performed in response to a male's courtship (nest coo) (Cheng et al 1998; Cheng 1992; Cheng 1986). Copulations that occur prior to the performance of nest coo do not lead to activation of the female reproductive system suggesting that female commitment is needed for egg laying (Cheng et al 1981). Nest coo behavior, therefore, may be a mechanism that allows for the prediction of the successful completion of a breeding cycle. Higher amounts of GNRH labeled cells, cells that are responsible for the hypothalamic-pituitary-endocrine response that initiates reproductive behaviors, have been found in doves during pre-laying stages of the breeding cycle than any portion of the breeding cycle following egg laying (Cheng et al 2011), validating this claim.

In this study, male doves that were sacrificed after being exposed to the egg laying phase of the breeding cycle had a marginally significant difference in positively labeled ZENK cells than those that were in the not bonded group. Because significant differences between positively labeled ZENK cells could be seen in the nest coo phase preceding egg laying in the breeding cycle, we expected doves that were exposed to egg laying to exhibit significant differences in cell counts to birds who were not bonded. We

suspect that marginal differences occurred in our study is due to small sample numbers and not to a change of state in the doves during this time period. Consistent with this idea, the number of cell counts within these group showed a similar trend. An additional study with a larger sample size is required to confirm this.

While exposure to the courtship phase of the breeding cycle has been found to initiate the process of pair bonding, allowing doves to form a preference for their mate, courtship behavior itself is not necessary to activate ZENK expression following bonding. Higher levels of ZENK labeled cells are present in the anterior taeniae when bonded doves are briefly exposed to their mate, without performing or observing courtship behavior (chapter 2), suggesting that the memory of the mate following initiation of the bond (once doves had undergone the nest coo phase) that is encoded in the taeniae. Bilateral lesions to the taeniae do not destroy nest coo behavior, but rather, doves that are taeniae lesioned perform greater amounts of it indiscrimately, namely, they court without regards to whether the animal they are courting to is their mate or unfamiliar, (chapter 2) reversing the effects of the bond. The present study does not address whether perception of nest coo alone or whether performing the act is critical for the formation of a bond. Further analysis of the nest coo stage during the breeding cycle must be done in or to address this question.

Chapter 4:

The nucleus accumbens and pair bonding in ring doves

The nucleus accumbens (NAcc) has been implicated in pair bonding in mammals (Young et al 2011; Young and Wang 2004; Bales et al 2007). In prairie voles, the existing animal model for mammalian pair bonding, dopamine receptors in then NAcc have been found to regulate partner preference and selective aggression, a species specific behavior involving aggressively attacking members of the opposite sex that the animal is not bonded to (Aragona et al 2006; Aragona et al 2003; Aragona and Wang 2004; Young and Wang 2004; Wang et al 1999). These studies have been the basis for human studies assessing the genetic components of monogamy (Garcia et al 2010; Walum 2008, Cherkas 2004).

Pair bonding in prairie voles, like in other bonded animals, is marked by preference for their mate. The average pair bond lasts for a period of 42 days, but can be disrupted following the death of one or both members of the pair or if the male leaves the home nest (Carter et al 1986). Preference for a mate is short-lived. Voles that are separated for a period of more than 24hrs will form new bonds. Bonding in both male and female voles initiates protection from aggressive attacks from the bonded mate (Carter et al 1986). Courtship behavior does not preceed bonding in prairie voles (Young 2003), but instead bond formation occurs following 24 hour period of cohabitation, during which time mating may not occur, but is hastened if the vole mates (Williams et al 1992). Pre-sexual priming behavior, nasogenital grooming, stimulates endocrine changes necessary for reproductive behaviors in voles (Carter et al 1986; Gavish et al 1983), however,

whether these behaviors are important for the initiation of bonding in voles has not been studied.

Like in prairie voles, pair bonding in ring doves is also measured by preference for the dove's mate (Morris and Erickson 1971). Although they stray, ring doves form lifelong pair bonds, retaining preference for their mate despite an extended period of separation. Morris and Erickson found that when doves have gone through the breeding cycle and had been separated for a period of several months, they recognize their mates amongst a colony of other doves and prefer to spend time in proximity to them (1971). Choosing to be near their mate is mimicked in laboratory based preference tests (Erickson 1973; Clavijo and Cheng 2007). We show that the entire breeding cycle need not be completed for a bond to be formed. Completing the courtship stage of the breeding cycle is sufficient to allow for the commencement of the bond (chapter 3). During the courtship stage, the hypothalamic-pituitary-endocrine reproductive system in female ring doves is stimulated by the female's own nest coo behavior, in response to nest coo solicitation from males.

The amygdala has been implicated in pair bonding in both prairie voles and ring doves (the homologous structure in doves is the nucleus taenaie). Lesions to the amygdala in prairie voles damaged a mate's protection against aggressive behavior (Demas et al 1997). Bilateral taeniae (avian amygdala) lesions damage preference for the dove's mate and disrupt pair bonding's moderation of courtship behaviors, specifically nest coo behavior (chapter 3). The mammalian amygdala and avian taneiae have projections to the nucleus accumbens, an area associated with rewarding behavior (Sah et

al 2003; Swanson and Petrovich 1998;Cheng et al 1999). Due to its connectivity to the nucleus taeniae and its involvement in mediating bonding associated reproductive behaviors in voles (Young et al 2011; Young and Wang 2004; Bales et al 2007), our current study seeks to assess whether the nucleus accumbens is also involved in mediating bonding behavior in ring doves. Preference for a mate before and following nucleus accumbens lesions will be assessed to determine if pair bond formation or maintenance is mediated by the nucleus accumbens. If the nucleus accumbens mediates bonding in doves, our study would suggest that this neural substrate, that has been found to regulate bonding in voles, may be conserved across species, despite vast differences in bonding behavior.

Experiment 1: Pair bond maintenance

Methods:

Subjects:

Sixteen male doves that were bred and housed in an AAALAC accredited animal care facility at Newark, Rutgers University were divided into two groups: a bonded and a non-bonded group. Doves in the bonded group were allowed to go through a breeding cycle, including the rearing of a squab. Doves in all groups were subjected to preference tests and behavior tests that assessed amount of courtship, underwent electrolytic bilateral lesions to the nucleus accumbens, and were tested for mate preference courtship behavior following recovery from surgery.

Electrolytic Lesions to Nucleus Accumbens:

Doves were anesthetized using Chloropent (2.5 ml/kg) before the initiation of surgical lesions. In addition, Marcaine (.0125%) was infiltrated subcutaneously along incision line prior to the lesion. The dove's head was fixed on a sterotaxic instrument at an angle of 45 degrees. A 0.03 mm electrode (O.D. tungsten wire) was inserted into the following coordinates: 6(A), 1.4(L), 4.7(V). Coordinates were derived from a modification of coordinates from a pigeon atlas (Karten and Hodos 1967) as described by Gibson and Cheng (1979). One milliamp of positive current was administered for 30 seconds. Doves were lesioned bilaterally. Doves were sutured following the procedure and allowed to recover. Two sham lesioned birds that were not bonded underwent the same procedure, as a control for the surgical procedure, but no current was administered at the time of the surgery. Males were housed alone following surgery and allowed to recover.

Behavioral Observations:

Male ring dove's behavior was tested before lesion and one week after lesion, to allow doves to recover from the surgery. Males were housed in isolation a day before testing. During the test day, males were put into a cage with an opaque divider that was inserted vertically down the middle of the cage. A female (either a familiar female the dove was housed with prior to the test or the pair bonded mate, depending on which group the male dove was in) was but on the other side of the divider. The divider was pulled and behavior was observed for a total of 1 hour. Wing flips, Cackles, Preening, Nest Coo, Bow Coo, Perch Coos, and Mount/Crouch were recorded during this allotted time frame.

Preference Test:

Doves in both groups were placed in y-shaped preference test developed by our laboratory (Clavijo and Cheng 2007) prior to and following lesion surgery, similar to procedures listed in chapter 1. Briefly, the male dove was allowed to roam the chamber for 15 minutes preceding data collection in order to get acclimated with the chamber. Afterwards, two were put into plexi-glass contained chambers opposite each other. The males in the pair bonded group were exposed to their mate in one chamber and a novel stimulus female in another chamber, while the birds in the non-pair bonded group were exposed to two novel stimulus females. The subject was put behind a plexi glass compartment that allowed it to view both females but not interact with them or roam through the chamber. It was held there for an additional 15 minutes. The plexi-glass containing the subject was then lifted and the subject was allowed to roam the chamber freely for an hour. During this time, we recorded time spent in neutral territory and the amount of time spent on each side of the preference container. A percentage of time spent with their mate was calculated by dividing total time spent with mate by total time spent stimulus birds and mate. Birds were determined to have a preference for their mate if they spent more than 60% amount of time with their mate. For tests following bilateral nucleus accumbens lesions, doves were tested for preference one week after behavior tests were performed.

Statistical Analysis:

General linear models (GLM) were run to determine whether bond status and lesion had an affect courtship behavior when doves were tested with a stimulus female

and percent of time spent with mate (or with one bird, if in the non-bonded group) during preference tests. Bond status and whether the behavior was performed before or after the lesion were used as factors during our analyses. Three separate models were run using bow coo, nest coo, and preference test results as dependent variables.

Lesion site analysis:

After doves went through the preference procedure, birds were sacrificed by a lethal injection of Chloropent and perfused intracardially with 0.9% saline followed by 4% paraformaldehyde in 0.1 M phosphate buffered saline. The brains were stored in a solution of 25% sucrose in 0.1 MPBS overnight. Brains were frozen using powdered dry ice and 30 micron sections of fixed tissue were cut. Tissue was stained with cresyl violet and lesion sites were checked (see figure 1 and 2).

Figure 1: Cumulative lesion site area

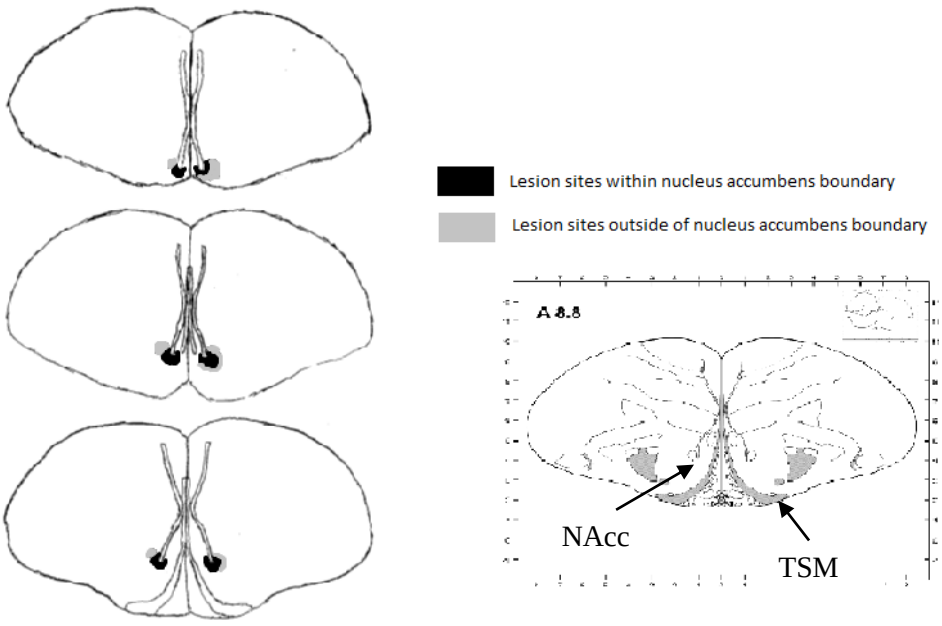


Figure 2: Image of lesion site

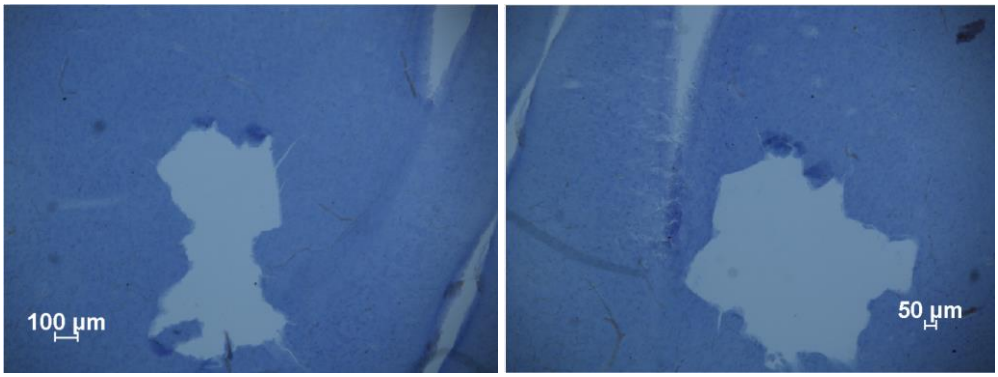


Figure 2: Image of lesion site (4x objective) for left (as seen on left) and right (as seen on right) lesion sites for one bird.

Results:

Nest coo:

Bond status ($F(1,30)=9.19$, $p=0.005$), but not whether behavior was performed before or after an NAcc lesion ($F(1,30)=0.085$, $p=0.773$) affected number of nest coos in doves. The interaction of bond status and lesion did not affect the amount of nest coos performed by the doves ($F(1,30)=0.957$, $p=0.336$). Whether doves were NAcc lesioned or were in the sham lesion group did not affect the amount of nest coo behavior ($F(1,30)=0.017$, $p=0.898$). There were differences in nest coo behavior ($_{MDiff}$=-49.03, SE=17.75, $p=0.01$) between doves that were bonded (M=61.69, SD=34.01) and non-bonded (M=111.75, SD=52.45). These differences were not impacted (M_{Diff}=-3.5, SE=19.3, $p=0.858$) by NAcc lesions, as there were no differences in nest coo behavior before (M=87, SD=65.48) and after lesions (M=92, SD=33.3). There were also no differences

in nest coo behavior (M_{Diff}=-21.93, SE=25.11, p=0.389) between doves in NAcc lesioned (M=87.06, SD=53.8) and sham lesioned groups (M=109 SD=13.4).

Figure 3: Number of nest coos before and after NAcc lesions

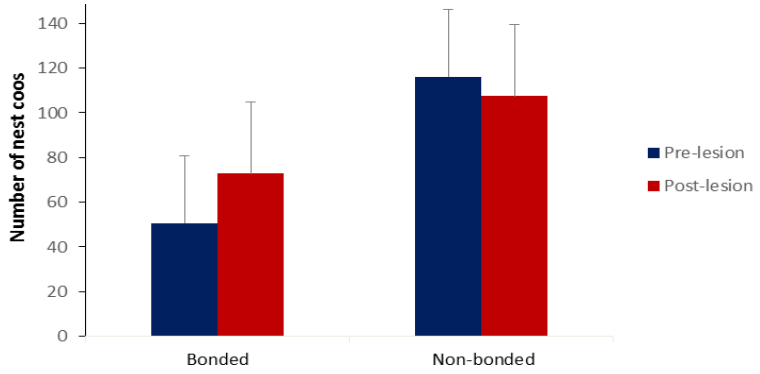


Figure 3: Number of nest coos before and after NAcc lesions in bonded and non-bonded groups.

Bow coo:

Neither bond status (F(1,30)=0.062, p=0.805) nor whether behavior was performed before or after lesion (F(1,30)=0.09, p=0.76) affected bow coo behavior in doves. There was no effect of type of lesion (sham lesion versus NAcc lesion) on bow coo behavior (F(1,30)=0.006; p=0.94). There were no differences (M_{Diff}=3.5, SE=19.72, p=0.86) between bow coo in doves in that were bonded (M=98.13, SD=54) versus not bonded (M=93.95, SD=47.9) . No differences (M_{Diff}=1.083, SE=21.47, p=0.96) in amount of bow coo was found in doves before (M=99, SD=50.13) or after (M=92.61, SD=52.9) NAcc lesions. There were no differences (M_{Diff}=0.063, SE=27.9; p=0.99) found in doves that had undergone sham lesions (M=95.75, SD=18.9) versus those who had undergone NAcc lesions (M=95.81, SD=52.84)

Figure 4: Number of bow coos before and after NAcc lesions

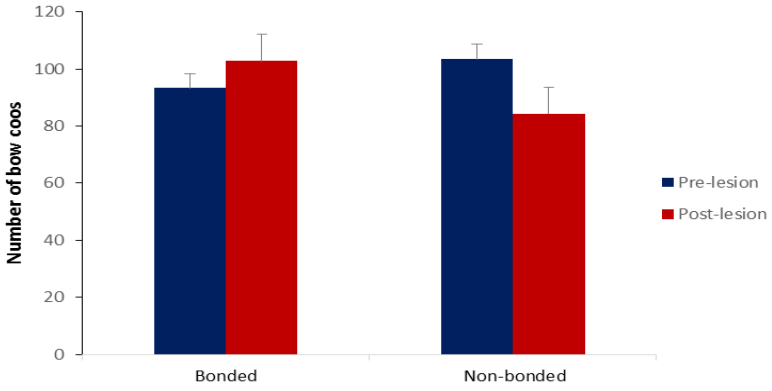


Figure 4: Number of bow coos before and after NACC lesions in bonded and non-bonded groups.

Preference:

Preference in doves was not affected by lesions to the NAcc ($F(1,30)=.737$, $p=0.398$), but was affected by bond status ($F(1,30)=18.98$, $p=.0001$). There was no effect of interaction of bond status and lesion ($F(1,30)=0.562$, $p=0.459$). There was no effect of type of lesion (sham vs. NAcc) on amount of preference ($F(1,30)=0.063$, $p=0.803$). There were differences ($M_{Diff}=.368$, $SE=0.086$, $p=.0002$) in percent of time spent with mate (or one female if dove were in the not bonded group) during preference tests in non-bonded ($M=0.42$; $SD=0.195$) and bonded ($M=0.787$, $SD=0.247$) NAcc doves. There were no differences ($M_{Diff}=0.065$, $SE= 0.093$; $p=0.493$) in percent preference in doves before ($M=0.59$, $SD=0.29$) and after lesions ($M=0.57$, $SD=0.28$). There were no differences ($M_{Diff}=0.208$, $SE=0.121$, $p=0.096$) in percent preference between of types of lesion, sham lesion ($M=0.57$, $SD=0.29$) or NAcc lesion ($M=0.59$, $SD=0.291$).

Figure 5: Percentage of time spent with mate

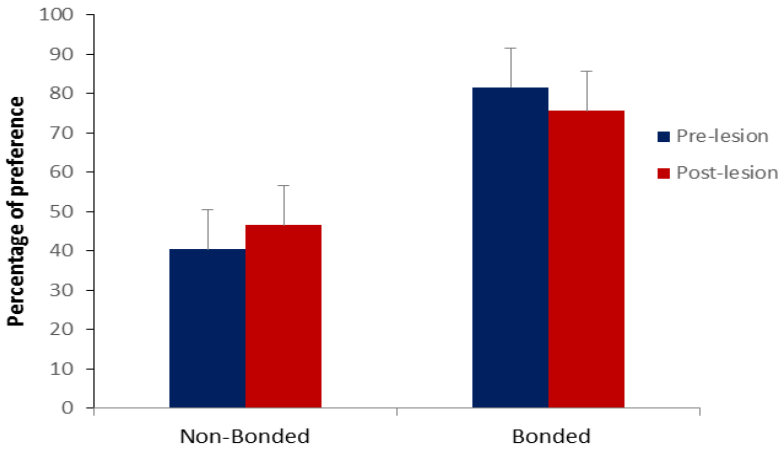


Figure 5: Percentage of time spent with mate (if bonded group) or a particular stimulus female (if non-bonded group) during laboratory preference tests before and after NACC lesions.

Experiment 2: Pair bond formation

Methods:

Subjects:

Ten male doves that were bred and housed in an AAALAC accredited animal care facility at Newark, Rutgers University and had never formed a pair bond were split into two groups: 1.)doves that were allowed to go through the breeding cycle, including the rearing of squab and 2.) doves that were housed with females but not allowed to go through the breeding cycle. Doves in all groups underwent an electrolytic lesion procedure, as described in experiment 1, which bilaterally damaged the nucleus accumbens. Two birds, that were not allowed to go through the breeding cycle, were sham lesioned to ensure that results were due to accumbens lesions versus effects of the surgical procedure. Doves in the group that were not allowed to go through a complete

breeding cycle were housed with a novel female that was switched every 3 days (to avoid the formation of a pair bond). All birds were subject to preference tests and behavior tests following recovery from surgery.

Housing procedures and spot checks:

Male doves were housed in isolation one week following surgery to allow them time to recover. After a week, doves that were not allowed to complete the breeding cycle and form a bond were housed with females which would be switched out every three days in order to prevent the formation of a pair bond. Doves that were allowed to go through the breeding cycle were housed with a female which remained in the cage with them until the end of the experiment. Doves were monitored daily to determine if they had met landmarks normally seen during the breeding cycle. For reference (Lehrman 1964; Lehrman 1959), bow coo will generally occur after the first day of pairing and nest coo behavior will occur after the second day of pairing. The female will lay her first egg between 1 week to 11 days after pairing and her second egg about a ½ a day to a day later. Eggs typically hatch about two weeks after the initial pairing and young are fed until they are 15-25 days old.

Preference Tests:

Doves in both groups were placed in a y-shaped preference test (asked described in experiment 1) developed by our laboratory (Clavijo and Cheng 2007) following a period of forty days (time it take after pairing for squab to reach maturity) to determine if preference for mate was observed following lesions to the nucleus accumbens. Birds

were determined to have a preference for their mate if they spent more than 60% amount of time with their mate.

Lesion site analysis:

Following the preference tests, doves were sacrificed and lesion sites were analyzed following procedures listed in experiment 1 (See figures 6 and 7).

Figure 6: Cumulative lesion site area

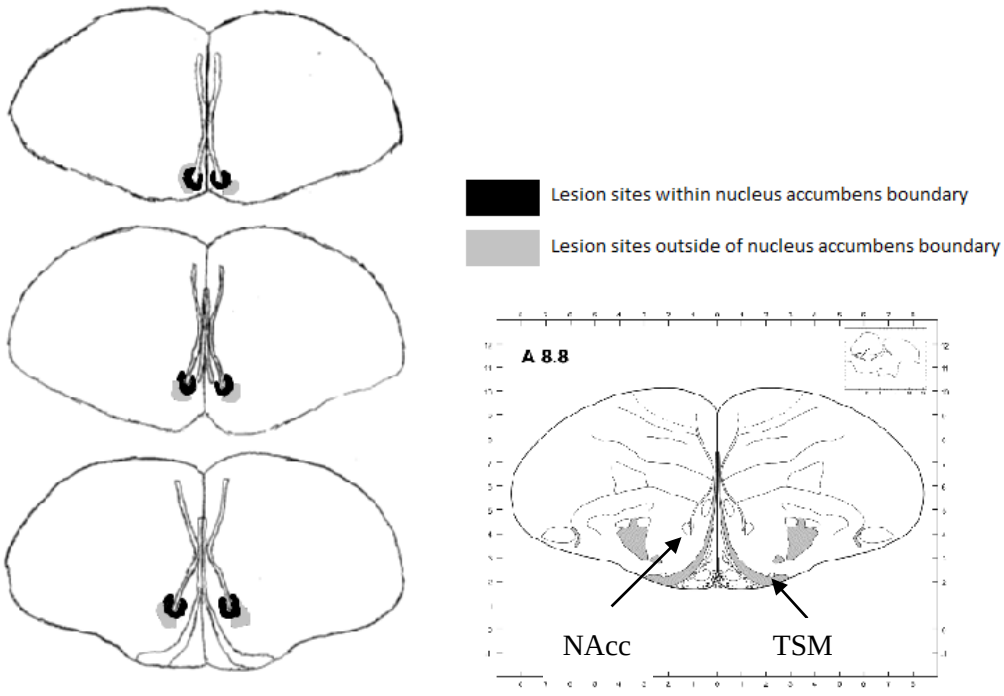


Figure 7: Image of lesion site

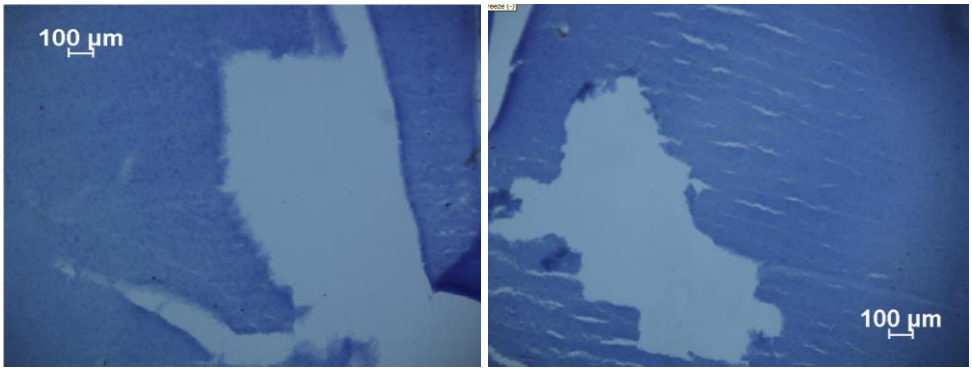


Figure 7: Image of lesion site (4x objective) for left (as seen on left) and right (as seen on right) lesion sites for one bird.

Results:

At the termination of the experiment (40 days), whether doves were allowed to go through the breeding cycle or not ($F(1,9)=33.4$, $p=0.0005$), but not lesion type ($F(1,9)=0.457$, $p=0.516$) had an effect on preference., Doves in the group that was allowed to go through the breeding cycle ($M=0.9$, $SD=0.091$) and those that were not allowed to go through the breeding cycle ($M= 0.43$, $SD=0.15$) differed ($M_{Diff}=0.46$, $SD=0.083$; $p=0.0004$) in the percent preference for their mate (or a novel female for group that was not allowed to bond). Doves in the sham lesion group ($M=0.49$, $SD=0.035$) did not differ ($M_{Diff}=0.172$, $SE=0.105$, $p=0.14$) in percent preference for their mate than those in the NAcc lesion group ($M=0.66$, $SD=0.295$).

Figure 8: Percentage of time spent with mate

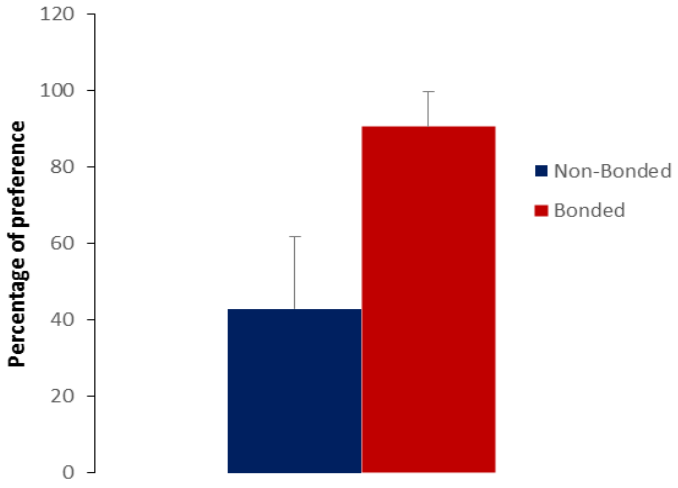


Figure 8: Percentage of time spent with mate (if in group that was allowed to go through the courtship cycle) or a stimulus female (if in group that was not allowed to go through the courtship cycle) following NACC lesions.

Nucleus accumbens lesioned doves in the group that were allowed to go through the breeding cycle met landmarks in the breeding cycle at similar times to animals that have not been lesioned (Lehrman 1964; Lehrman 1959). All animals performed bow coo behavior with the first day of exposure to a new female. On average doves performed nest coo behavior 1.6 days after exposure to female (SD=0.54). The female stimuli housed with lesioned males that were allowed to go through the breeding cycle laid eggs an average 9 days (SD=2.12) after exposure to the males and squab rearing took place an average of 14.2 days (SD=1.79) after birds were housed together.

Discussion:

The current model for pair bonding in mammals (Young et al 2011;Young 2003), the prairie vole, suggest that bonding related behaviors (i.e. aggression) are mediated by the nucleus accumbens. Our present study found that in ring doves, preference for a mate,

as measured by percentage of time spent with the mate versus another dove, and lower courtship behavior towards a mate in doves that were bonded as compared to doves that have not formed a bond were not altered by lesions to the nucleus accumbens. Doves that were paired following lesions to the nucleus accumbens were able to form pair bonds. These lesioned doves met all the landmark behaviors of the breeding cycle and developed a preference for their mate during laboratory based preference tests. These observations seem to suggest that aspects of bonding in different species, voles and doves, may be mediated by different neural substrates. In the following section, we will examine this.

Behavioral patterns leading to bonding differ in prairie voles and doves. While behaviors that lead to bond formation have not been assessed in voles, doves rely on a long phase of coordinated courtship behavior that is contingent on close interactions between males and females, a key to escalation of female nest-coo behavior that triggers reproductive endocrine response (egg laying) (Cheng et al 2011). In doves, copulations resulting from periods that are too early in the breeding cycle will not result in fertilizations (Cheng et al 1981). Males require some period of courtship before being receptive to females (Zenone et al 1979; Erickson 1986; Silver et al 1973; Silver 1978; Silver and Barbiere 1977). Females will not be responsive to males if courtship behavior is not directed at them (Friedman 1977). The courtship phase of breeding cycle lasts 7-11 days, before the females lay eggs (Lehrman 1964; Lehrman 1959). Mating in prairie voles happens much more quickly. Pair bonding will occur during the first six hours of meeting, if copulations occur. If they do not, behaviors associated with cohabitation for 24 hours, in voles, initiate bonding (Williams et al 1992). Although voles exhibit pre-coupulatory behaviors, such as nasogenital grooming (Carter et al 1986; Gavish et al

1983), which can be construed as a form of courtship, it is short lived and whether these behaviors are involved in bond formation has not been tested. Bonds are also broken much more rapidly in prairie voles. A day of separation, which generally involves males leaving the nest site, allows for females to re-mate (Carter et al 1986). Doves will retain preference for their mate for a period of 7 months (Morris and Erickson 1971) and, although longer separation periods have not been tested, it is possible that preference is retained for periods longer than this. Long periods of social interaction in doves, on the one hand, versus short periods of sexual interaction in voles provide a contrasting path to the formation of bonding. Sexual behavior is executed at the level of the hypothalamus, but the hedonic aspect of this act is fittingly represented by the rewards system, in particular the nucleus accumbens. Copulation alone does not lead to bond formation in doves. In contrast, copulation can quicken bond formation in voles suggesting that the nucleus accumbens could regulate hedonic aspect of sexual behavior in voles.

In both ring doves and prairie voles the nucleus taeniae (amygdala in mammals) has been implicated in pair bonding (Demas et al 1997, chapter 2). Lesions to the nucleus taeniae destroy preference for a mate and differentially directed nest coo behavior toward the mate, elevating levels of overall courtship behaviors towards doves which they are not mated to (chapter 2). In voles, estrus is induced by pre-sexual behavior, nasogenital grooming. Grooming lifts a pregnancy block and allows females to be fertilized. Amygdala lesions in prairie voles alter pair bonding's protection from pregnancy block, suggesting that amygdala may play a role in mate recognition and preference in voles (Demas et al 1997). Mate recognition, which is a necessity for

animals that use pair bonding as a reproductive strategy, is governed by the nucleus taeniae in both species, an area associated with survival driven emotional behaviors.

Although copulatory behavior is not necessary for pair bond formation in doves, ring doves clearly partake in this behavior. Although damage to the nucleus accumbens did not dissolve preference for a mate or prevent mates from forming pair bonds, this region may be involved in regulating aspects of sexual behaviors in birds. Assessment of immediate early gene activation (Fos) in the nucleus accumbens in quail have found increases in positively labeled cells when males participated in sexual behaviors (Tlemcani et al 2000). Immediate early gene ZENK was selectively positively labeled in male pigeons when they were exposed to a female pigeon (presumably, these birds were not bonded) versus when they were exposed to an empty cage (Husband 2004). While no differences were found in ZENK activation between doves that were bonded and those that were not bonded, ZENK labeling is present in the nucleus accumbens in ring doves after exposure to females (chapter 1). Whether presence of ZENK labeled cells was due to participation in reproductive (sexual) behaviors or whether it was at background levels is not known. The present study showed that bilateral nucleus accumbens lesions did not affect courtship behavior; however, analysis of copulatory behavior could not be done properly due to low numbers of mounts and mounting attempts during our behavior tests. Assessing whether damage to the nucleus accumbens affects copulatory behaviors will provide further insight into the nucleus accumbens' role in reproductive behavior.

Chapter 5

The impact of pair bond status on the rate of neurogenesis induced by VMN damage

Introduction:

The production of new neurons occurs naturally in several regions of the adult brain, including the most widely studied mammalian hippocampus (Gage et al 1998; Gould et al 1999), olfactory system (Lois and Alvarez-Buylla 1994), and high vocal system of song birds (Nottobohm 1985). Adult neurogenesis can also occur in response to brain perturbation. In ring doves, adult neurogenesis has been shown to occur in the ventral medial nucleus of the hypothalamus (VMN) (Cao et al 2002), an area that does not normally produce new neurons (Nottobohm 2002). The destruction of the VMN initiates the recruitment of new neurons from the sub-ventricular zone (SVZ) after a period of 7-14 days (Cao et al 2002). Following a recovery period of eight weeks, the damaged region takes on various functions lost post lesion, allowing for courtship behavior to re-emerge (Chen et al 2006; Cheng 2013).The VMN mediates the production of nest coo behavior (Cheng 1992; Gibson and Cheng 1979; Bernstein et al 1993) and bilateral VMN lesions impair the production of the nest coo call (Gibson and Cheng 1979; Bernstein et al 1993). The importance of this courtship behavior is demonstrated in its ability to trigger hormonal changes necessary for the follicular maturity that leads to ovulation (Cheng 1986; Cheng et al 1998; Cheng 1992) via its input from the mid-brain auditory nucleus (mICo) and from the thalamic auditory nucleus (Ov) (Durand et al 1992; Cheng and Zuo 1994). Electrophysiological studies identify that cells in the VMN selectively respond to female nest coo stimulation. Activation of these cells result in the hypothalamic-pituitary reproductive endocrine response (Cheng et al 1998)

In mammals, social factors such as isolation (Stranahan et al 2006) and chronic stress (Joels et al 2007) have been known to affect neurogenesis. In ring doves, factors such as housing conditions are involved in the facilitation of neurogenesis in the VMN and recovery of courtship function. Production of differentiated new neurons and functional recovery of courtship behavior after a VMN lesion in male doves is increased in doves that are housed with females versus those housed with males (Chen et al 2006). Doves housed in isolation were not able to recover behavior (Chen et al 2006), suggesting that exposure to stimuli, in the form of other doves, may be significant in the recovery process, however, much is still unknown about the nature of the housing condition that is driving rate of neurogenesis in the VMN. Differences in the amount of differentiated new neurons in the VMN when male doves are exposed to birds of different sexes, suggest that this process is sensitive to the type of stimuli the doves are exposed to. Social interactions, however, are not limited to the sensory components of the stimulus, but are also subject to effects of the interaction itself and motor behavior produced by the animal. Disparities in the rate of neurogenesis could, therefore, be attributed to several factors that have not been assessed during previous studies. Testing doves of different bond statuses, thereby exposing doves to stimuli that vary in social meaning, but not in type of sensory input, may provide a means for assessing whether motor behavior, the nature of the stimulus properties, or interaction effects play a role in these differences by. Intensity of courtship behavior (nest coo), a behavior that is regulated by the VMN, is moderated by bond status (chapter 2). Doves that have not entered into a pair bond perform higher amounts of courtship behavior than doves that are pair bonded do towards their mate (chapter 2, study 2), allowing for a means to test

whether differences in the subject's motor behavior have an effect on the amount of newly differentiated cells in the VMN. Additional lesions to the nucleus taeniae (avian amygdala), the region encoding memory of a pair bonded mate (chapter 2, study 1), will provide a further confirmation for the effects of bond status on rate of neurogenesis.

Our current study seeks to understand the impact of an emotional social factor, pair bonding, on the amount of new neurons produced in the VMN after lesion and recovery of courtship behavior and address whether lesions to the nucleus taeniae, which eliminate memory of a bonded mate, impact the amount of lesioned induced neurogenesis in the VMN and courtship behavior during the recovery period following VMN lesions. Male doves will be housed with females whom they are bonded to (familiar females) and those whom they are not bonded to (unfamiliar females) to determine if the amount of new neurons occurs at a higher rate in either of these cases. Additionally, we will address whether the female stimuli's behavior, attributed to bonding, has an effect on the recovery of the male's courtship behavior by assessing the female's behavior during behavior tests when males are tested with females they are housed with and by providing an additional study which exposes all males to the same type of stimulus female during behavior tests. We suspect that males have a higher number of differentiated new neurons and will perform more courtship (nest coo) behavior when they have not undergone bonding during the recovery process following VMN lesion, in keeping with behavior witnessed before they were lesioned (chapter 2, study 2), irrespective of the female they are tested with during behavior tests.

We will analyze the effect of damaging the nucleus taeniae on neurogenesis and recovery of function after VMN lesion (a double lesion study), to confirm whether bond

status has an effect on this process by erasing the memory of a mate. We hypothesize that, if bond status affects the amount of neurogenesis and behavioral recovery in the VMN, damaging the taeniae will reverse the effects of bonding, thereby eliminating preference for a mate and bonding's moderation of courtship behavior.

Summary of Experiments:

Experiment Number	Explanation
1	Determines whether bonded/non-bonded doves housed with mates/familiar females, respectively, influence the rate of neurogenesis and courtship behavior when tested with a stimulus female (mate or familiar female) during recovery period following VMN lesion. Only the male's courtship behavior is measured.
2	Assesses female behavior and male's behavior unrelated to the breeding cycle during recovery period following lesion to determine if it affected subject's courtship behavior during behavior tests.
3	Controls for effect of female stimulus during behavior tests on the rate of neurogenesis and courtship behavior during the recovery period following VMN lesion by exposing all males to novel females during behavior tests, regardless of which female they are housed with during the experiment.
4	Confirms the effect of bond status on the rate of neurogenesis and courtship behavior during recovery period following VMN lesions by assessing whether lesions to the taeniae, an area that mediates memory for a pair bonded mate, reverses the effects of being housed with a bonded mate on rate of neurogenesis and courtship behavior during recovery period following VMN lesion.

Experiment 1: Effect of housing stimulus (female) of varying bond status on VMN lesion induced new neuron production and behavior during recovery period following lesion.

Methods:

Subjects:

Twelve male doves that were bred and housed in an AAALAC accredited animal care facility at Newark, Rutgers University were equally divided into two groups: a bonded group (n=6) and a non-bonded group (n=6). Two additional birds were used for sham lesions to ensure that results were not due to surgical procedure. The bonded group was allowed to mate with a female and rear at least one squab, as described by Morris

and Erickson (1971). The non-bonded group and bonded group were housed separately a week prior to the initiation of the experiment. During the experiment, the non-bonded group was housed with stimulus females that were rotated out of their cage on Monday, Wednesdays, and Fridays of every week in order to prevent the possibility of them bonding.

Electrolytic VMN Lesions:

Lesions followed procedures described by Chen et al (2006). Briefly, doves were anesthetized using Chloropent (2.5 ml/kg) before the initiation of surgical lesions. In addition, Marcaine (.0125%) was infiltrated subcutaneously along incision line prior to the lesion. The dove's head was fixed on a sterotaxic instrument at an angle of 45 degrees. A 0.03 mm electrode (O.D. tungsten wire) was inserted into the following coordinates: 2.6 mm anterior to the interaural line (L), 0.5 mm lateral to the midline (DV), and 8.5 mm ventral to the dura. One milliamp of positive current was administered for 30 seconds. Doves were lesioned bilaterally, sutured, and allowed to recover. Sham lesioned birds underwent the same procedure, but no current was administered at the time of the surgery.

BrdU injections:

In accordance to procedures done previously in our lab (Chen et al 2006) the doves were given daily injections of BrdU from the first to the sixth post-operative day. Doves were injected with 50 mg/kg weight intraperitoneally. BrdU was dissolved in 0.09% NACL at a concentration of 10mg/ml and was filtered.

Behavioral Observations:

Male ring dove's behavior was tested at several time points after the lesion surgery in accordance with procedures done previously in our lab (Chen et al 2006). Briefly, males were housed in isolation a day before testing. During the behavior test day, males were put into a cage with an opaque divider that was inserted vertically down the middle of the cage. A female (either familiar female the dove was housed with prior to the test or the pair bonded mate, depending on which group the male dove was in) was put on the other side of the divider. The divider was pulled and the male's behavior was observed for a total of 1 hour. Wing flips, Cackles, Preening, Nest Coo, Bow Coo, Perch Coos, and Mount/Crouch were recorded during this allotted time frame. Behavioral observations for lesioned pair bonded and non-bonded birds were done at a baseline (pre-lesion), 1 week post-lesion, 4 weeks post-lesion, 6 weeks post-lesion, and 8 weeks post-lesion time frame.

Preference Test:

Doves in both groups were put through a y-shaped preference test developed by our laboratory (Clavijo and Cheng 2007). The male dove was allowed to roam the chamber for 15 minutes preceding data collection in order to get acclimated with the chamber. Afterwards, two were put into plexi-glass contained chambers opposite each other. The males in the pair bonded group were exposed to their mate and a novel stimulus female, while the birds in the non-pair bonded group were exposed to two novel stimulus females. The subject was put behind a plexi glass compartment that allowed it to view both females but not interact with them or roam through the chamber. It was held

there for an additional 15 minutes. The plexi-glass containing the subject was then lifted and the subject was allowed to roam the chamber freely for an hour. During this time, we recorded time spent in neutral territory and the amount of time spent on each side of the preference container. A percentage of time spent with their mate was calculated by dividing total time spent with mate by total time spent stimulus birds and mate. Birds were determined to have a preference for their mate if they spent more than 60% amount of time with their mate. Doves were anesthetized using Chloropent (2.5 ml/kg) and sacrificed an hour after the preference test via perfusion.

Immunoflorescence:

A similar procedure to that used in Chen et al (2006) was employed for Immunoflorescense procedures. Briefly, Birds were sacrificed by a lethal injection of Chloropent and perfused intracardially with 0.9% saline followed by 4% paraformaldehyde in 0.1 M phosphate buffered saline. After being fixed overnight, the brains were stored in a solution of 25% sucrose in 0.1 MPBS overnight. Brains were frozen using powdered dry ice and 30 micron sections of fixed tissue were cut. After two rinses in 10 mM PBS each for 10 min, sections were incubated in 2 N HCl at 37 degrees Celsius for 30 min, followed by two 5 min rinses in 0.1 M boric buffer, pH 8.5, and then two 5 min rinses in PBS. The sections were then incubated overnight at in PBS with a mixture of primary antibodies including rat anti-BrdU (1:200), a marker for newly proliferated cells, and mouse anti-NeuN (1:200), a marker for mature neurons. After two 10 minute rinses in PBS, the sections were incubated in a solution of biotin-conjugated donkey anti-rat (1:200) and Alexa 488-conjugated donkey anti-mouse IgG (1:250) for 90

minutes, followed by streptavidin-conjugated Alexa 568 (1:250) for 90 minutes. Sections were mounted onto slides and cover slipped.

Cell Counting:

Nikon Diaphot fluorescence microscopy and confocal laser-scanning microscopy (Bio-Rad 1024 system) was used for all cell counting to insure that staining for both antibodies were colocalized on the same cell. The z step was set at 1 micron or less for z-series analysis. The 3-D confocal images were assembled using Metamorph software. In addition Image J software was used to weave images of the same section along multiple z-planes together in order to allow for smoother cell counting. A total of three sample sections were imaged at 20X for each bird and number of positively labelled cells from each these sections were averaged. Convergence of Brdu and NeuN label on the same cell suggests that the cell (double labeled cell) is a mature neuron. A confocal laser-scanning microscope was used for **all** cell counting to insure that staining was colocalized on the same cell. All cells that were positively labeled with both BrdU (in the nucleus) and positively labeled with NeuN (in the cell) were accepted to be double labeled cells. Although, for the most part, lesions had closed following the 8 week recovery period, sites were verified by the location of immunofluorescence staining (see figures 1 and 2).

Results:

Lesion sites:

Figure 1: Cumulative lesion site area

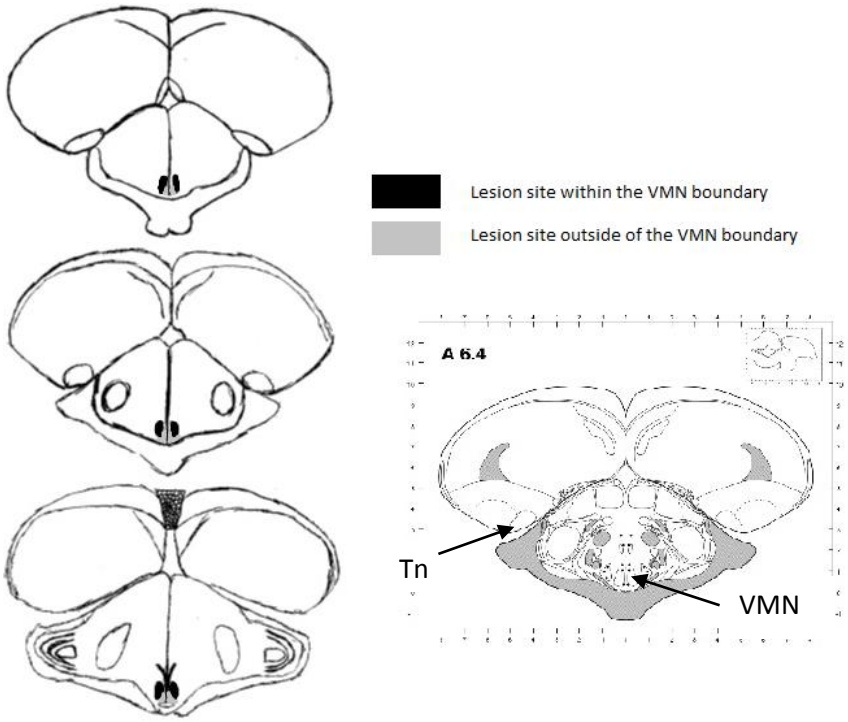


Figure 2: Double labeled cell within the VMN

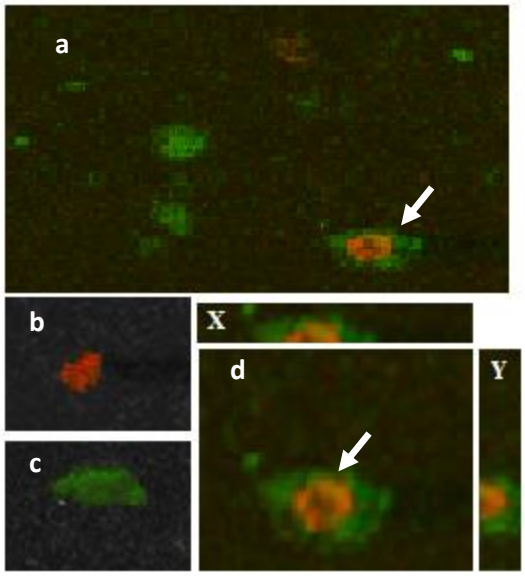


Figure 2: An image of a merged double label BrdU+ NeuN cell taken from the VMN using a confocal microscope (a) . The red labeled cell represents a cell that is positively labeled for BrdU, a new born cell marker (b) while the green labeled cell represents a cell that is positively for NeuN, a mature neuron marker (c). Both labels exist within the same cell (d) and, therefore, represent a new born neuron.

Effect of bond status on double labeled cells and courtship behavior:

Males that didn't form a pair bond (M=36.33; SD=24.08) had significantly more positively labeled BrdU/NeuN cells (t (10)=2.57, p=0.014) than doves that were in the bonded group (M=9.33; SD=8.7) (See figure 3). Male doves in the non-bonded group displayed a higher amount of courtship behavior (t(10)=2.3; p=0.02) towards non-bonded females (M= 156.5; SD=49.4) than males in the bonded group did to their mates (M=102; SD=31.8) (See figure 4). Amount of nest coo behavior was positively correlated with the number of positively labeled BrdU/NeuN cells in the VMN (r (10) =.799, p=0.002). There was no difference in the number of single labeled BrdU (t(10)=0.18, p=0.43) and NeuN (t(10)= 0.34, p=0.37) cell counts between doves that are bonded (BrdU: M=83; SD=11.2; NeuN: M=55; SD=14.7) and not bonded (BrdU:M=80; SD=16.7; NeuN: M=59.2; SD=13.2). For each male dove, no behavior was performed 1 week post lesion. In addition, behavior seemed to increase dramatically at week 4 for males in the non-bonded group.

Figure 3: Number of double labeled BrdU/NeuN cells

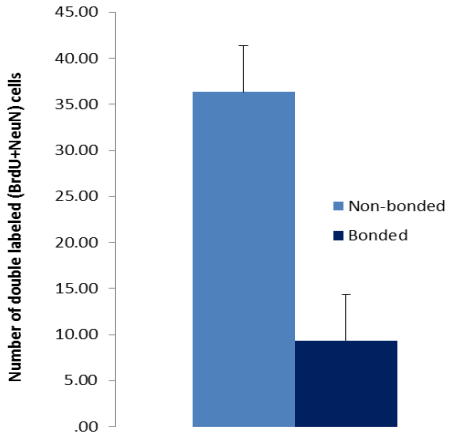


Figure 3: Number of double labeled cells (BrdU+NeuN) found in doves in bonded and non-bonded group.

Figure 4: Amount of nest coo behavior

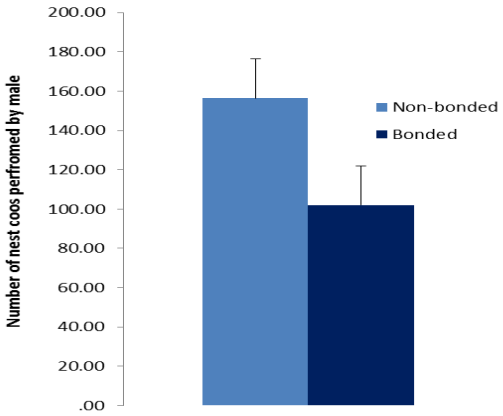


Figure 4: Number nest coos performed by doves in bonded and non-bonded group.

Effects of bond status on preference:

Preference tests between bonded (M=80.8, SD=15.7) and non-bonded groups (M=55.5, SD=11.4) indicate significant differences between percentage of time spent with mate (or stimulus bird for non-bonded group) (t(10)= 3.2, p=0.004) (See figure 5).

Figure 5: Percentage of time spent with mate

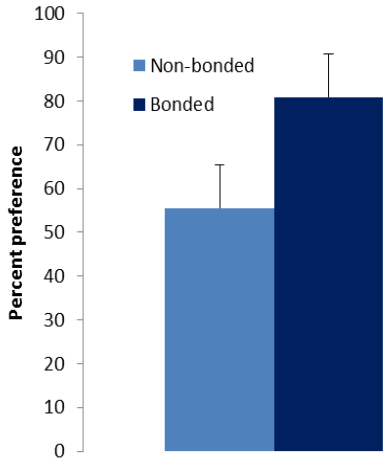


Figure 5: Percentage of time spent with mate (bonded group) or a novel female (non-bonded group) during laboratory preference tests.

Experiment 2: Male's courtship behavior during recovery period following VMN lesion: effect of female's behavior during testing period

Methods:

Subjects:

The subjects were a group of twelve male ring doves bred and housed in an animal housing facility in the AAALAC accredited animal care facility at Newark, Rutgers University. The doves were equally divided into two groups: a bonded group (n=6) and a non-bonded group (n=6). The bonded group was allowed to mate with a female and rear at least one squab, as described by Morris and Erickson (1971). The non-bonded group and bonded group were housed separately a week prior to the initiation of the experiment. During the experiment, the non-bonded group was housed with stimulus females that were rotated out of their cage on Monday, Wednesdays, and Fridays of every week in order to prevent the possibility of them bonding. Electrolytic lesions were done as described in experiment one.

Behavioral Observation:

Males were housed in isolation a day pre-testing and were subjected to a similar type of test as they were in experiment one. During test day, males were put into cages with opaque dividers with their mated female (if they were in the bonded group) or the familiar female with whom they were housed with (if they were in the non-bonded group) prior to the test, the dividers were lifted and behavior was observed for one hour, as detailed in experiment 1. In addition, female behavior was recorded during the testing

session to determine amount of courtship behavior produced by the stimulus. Wing flips, cackles, preening, nest coos, perch coos were recorded during this allotted time frame. Behavioral observations for lesioned pair bonded and non-bonded birds and their stimuli were done at a baseline (pre-lesion), 1 week post-lesion, 4 weeks post-lesion, 6 weeks post-lesion, and 8 weeks post-lesion time frame. In addition, other types of behaviors including pecking, eating, walking, and drinking were measured. A preference test (described in experiment one) was conducted at week 8 to determine if doves retained a preference (attachment) for their pair bonded mate. Doves were sacrificed an hour following the preference test. Brains were extracted, immunolabeled, and counted in accordance to procedures listed in experiment one.

Results:

Effects of bond status:

A general linear model (glm) was used to determine if bond status had an effect on nest coo behavior after VMN lesions across the 8 week testing period. Recovery week and bond status were used as factors in this experiment and female behavior and other behaviors were used as covariates. Nest coo data was transformed using a $\ln(x+1)$ to correct for differences in variance across groups while allowing for the maintenance of "0" value data (Fletcher et al 2005). The male's bond status ($F(7,40)=4.38$, $p=0.43$) and the week the behavior was performed ($F(7,40)=4.15$, $p=012$) had an effect on our model. Female behavior had no effect on male's performance of nest coo behavior ($F(7,40)=0.138$, $p=0.712$). Other behavior did not have an effect on nest coo behavior ($F(7,40)=0.65$, $p=0.8$).

Individual tests assessing differences in male nest coo behavior across testing time points. Nest coo behavior was performed at a significantly higher amount in doves that were not bonded (week1: M=5.8, SD=5.9; week 4: M=107, SD= 119; week 6: M=32.7, SD=44.5; week 8: M=89, SD=76.2) versus those that were bonded(week 1: M=1.7, SD=2.7, week 4: M= 18.3, SD=21.5; week 6: M= 4.17, SD=44.5; week 8: M=21, SD=18.5) during weeks 4(t(10)=1.8, p=0.05) and 8(t(10)=2.12, p=0.03, but only marginally so in week 6 (t(10)=1.56, p=0.08) and week 1 (t(10)=1.5, p=0.07) (See figure 6).

Figure 6: Number of nest coos performed by males

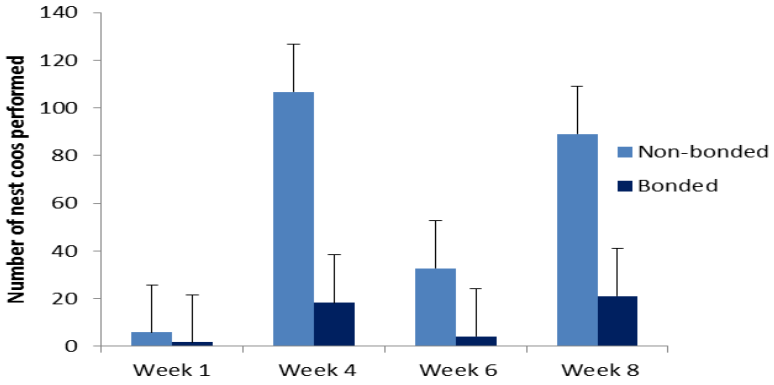


Figure 6: Number of nest coos performed by males during recovery period after VMN lesion during behavior tests.

Female nest coo behavior over eight weeks (figure 7) was also assessed. During the first (t (10)=1.103, p=0.3), sixth (t=1.48, p=0.17), and eight (no nest coo behavior performed) week of behavioral testing, females tested in either the bonded (week1:M=0.67, SD=1.03; week 6: M=3.33, SD=0.33) or non-bonded group (week 1: M= 0.17, SD=0.41; week 6: M=0.33, SD=0.82) showed equal amounts of nest coo

behavior. During week four (t(10)=2.27, p=0.047), females in the bonded group (M=33.3,SD=36) showed higher amounts of nest coo behavior than those in the non-bonded group (M=0; SD=0).

Other behaviors (walking, pecking, eating seeds, etc.) were measured across behavioral test weeks. At week 1, doves in the bonded group (44.8, SD=15.9) performed more other behaviors ((t (10) =3.02, p=0.01) than those that were the non-bonded group (M=20.3; SD=11.8). At week 4, the pattern switched so that the non-bonded group (M=66.6; M=20.6) performed more amounts of other types of behavior than the bonded group (42.50), although this switch was not significant (t (10) =1.64, p=0.15). At week 6, the doves in the bonded group (M=119.5, SD=35) performed more amounts of other behavior (t (10) =2.93, p=0.017) than those in the non-bonded group (M=72, SD=18). The direction of significance switched, again, at week 8 (t (10) =3.15, p=0.01) when the non-bonded group (M=136, SD=57.6) performed more behavior than the bonded group (M=58, SD=19.1).

Figure 7:Number of nest coos performed by females

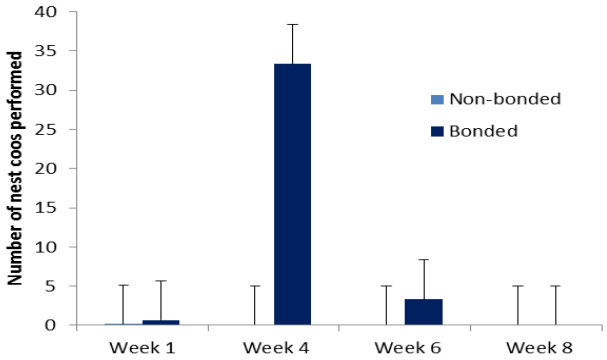


Figure 7: Number of nest coos performed by females housed with males that underwent VMN lesions at the time of behavior tests during recovery period following lesion.

Preference tests:

Preference tests at the 8 week recovery time point indicate that males in the bonded group prefer to spend a greater percentage of time (t(10)= 9.627, p=0.0001) with their mate (M=96.1; SD=6.2) than non-bonded doves prefer to spend with a stimulus female (M=51.7, SD=9.4) (See figure 8).

Figure 8: Percentage of time spent with mate

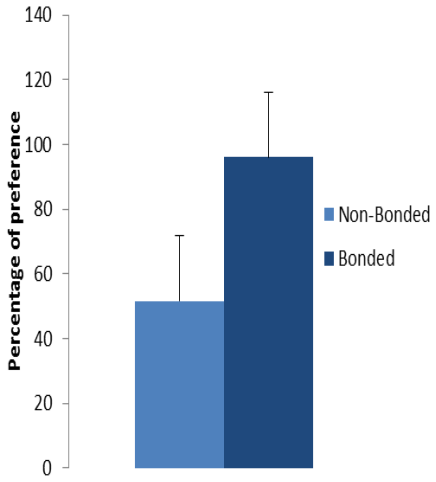


Figure 8: Percentage of time animals preferred to spend with a mate (if in bonded group) or a novel female (if in non-bonded group)

Experiment 3: Effect of standardizing testing stimulus (exposing all males to a novel female during behavior tests) on neurogenesis, recovery of courtship function, and mate preference

Methods:

Subjects:

Twelve male doves that were bred and housed in an AAALAC accredited animal care facility at Newark, Rutgers University were equally divided into two groups: a

bonded group (n=6) and a non-bonded group (n=6). Two additional birds were used for sham lesions to ensure that results were not due to surgical procedure. The bonded group was allowed to mate with a female and rear at least one squab, as described by Morris and Erickson (1971). The non-bonded group and bonded group were housed separately a week prior to the initiation of the experiment. During the experiment, the non-bonded group was housed with stimulus females that were rotated out of their cage on Monday, Wednesdays, and Fridays of every week in order to prevent the possibility of them bonding. Electrolytic lesions and BrdU injections were done in accordance to procedures in experiment 1.

Behavior tests:

Males were housed in isolation a day prior to testing and were subjected to a similar type of test as they were in experiment one. During test day, males were put into cages with opaque dividers, with a novel stimulus female on the other end of the divider, the dividers were lifted and behavior was observed for one hour, as detailed above. In addition, female behavior was recorded during the testing session to determine amount of courtship behavior produced by the stimulus. Wing flips, Cackles, Preening, Nest Coo, Perch Coos were recorded during this allotted time frame. Behavioral observations for lesioned pair bonded and non-bonded birds and their stimuli were done at a baseline (pre-lesion), 1 week post-lesion, 4 weeks post-lesion, 6 weeks post-lesion, and 8 weeks post-lesion time frame. A preference test (described in experiment one) was conducted at week 8 to determine if doves retained a preference (attachment) for their pair bonded

mate. Doves were sacrificed an hour following the preference test. Brains were extracted, immunolabeled, and counted in accordance to procedures listed in experiment one.

Results:

We ran a glm assessing differences in in the amount of double labeled cells (NeuN+BrdU) in the VMN (outcome variable) depending on bonding status. We used amount of nest coo behavior for males and stimulus females as covariates and included week nest coo was performed as a factor. Nest coo data was transformed using an ln(x+1) to correct for differences in variance across groups while allowing for the maintenance of "0" value data (Fletcher et al 2005). Our model showed a difference between doves that are pair bonded versus those that are not pair bonded in the amount of courtship behavior produced ($F(7,40)=67.8$, $p=0.00$). There was no effect of male nest coo ($F(7,40)=0.488$, $p=0.489$) or stimulus female nest coo ($F(7,40)=0.463$, $p=0.5$).

When not taking into account other factors, there is a significantly greater number of double labeled cell counts in the VMN ($t(10)= 4.6$, $p=0.001$) in non-bonded doves ($M=54.5$; $SD=13.9$) than in bonded doves ($M=23.5$; $SD=8.74$) (See figure 9).

Figure 9: Number of double labeled BrdU/NeuN cells

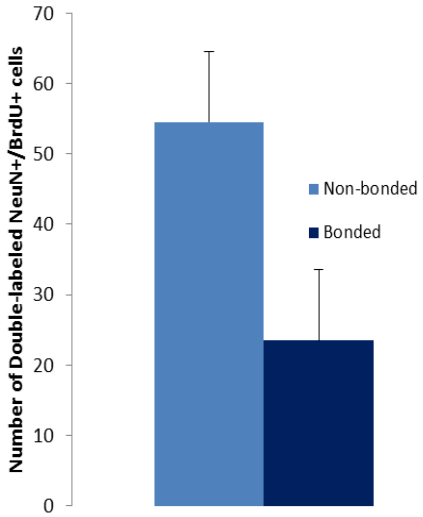


Figure 9: Number of double labeled (BrdU+NeuN) cells following eight week recovery period when male doves were exposed to a novel female during behavior tests (regardless of housing condition).

Nest coo behavior was greater in doves that were not bonded versus those that were bonded (See figure 10). During the first week following VMN lesions, individual tests in male doves showed no difference ($t(10)=0.62$, $p=0.27$) in nest coo behavior regardless if they were in the bonded ($M=0.33$; $SD=0.51$) or non-bonded group ($M=0.17$; $SD=0.41$). During weeks 4($t(10)=2.98$, $p=0.007$), 6,($t(10)=2.136$, $p=0.02$), and 8 ($t(10)=3.057$, $p=0.006$) doves in the non-boned group (week 4: $M=74$, $SD=21.6$; week 6: $M=117.3$, $SD=44.8$, $M=113.8$, $SD=20.7$) showed greater amounts of courtship behavior than those in the bonded group (week4: $M=36$, $SD=22.5$; week 6: $M=77.3$, $SD=12.3$; week 8: $M=83.3$, $SD=12.9$).

Figure 10: Number of nest coos performed by males

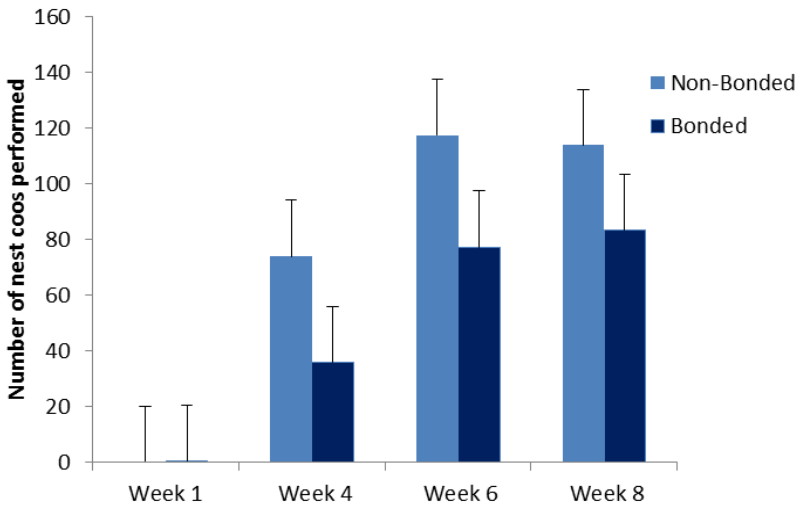


Figure 10: Number of nest coos performed across eight week recovery period when male doves were exposed to a novel female during behavior tests (regardless of housing condition).

Stimulus females during the first week did not perform any type of nest coo behavior, regardless of which group they were in. During the 4th week, females in the non-bonded group (M=0.167, SD=0.41) did not have significantly different (t(10)=0.45, p=0.66) amounts of courtship behavior than those in the bonded group(M=0.33; SD=0.81). Nest coo behavior also did not differ between bonded (week 6: M=1, SD=2 week 8: M=0) and non-bonded (week 6: M=0.33; SD=0.81; week 8: M= 0.33, SD=0.81) group females during weeks 6 (t(10)= 0.76, p=0.47) and 8 (t(10)=1, p=0.34) (See figure 11).

Figure 11: Number of nest coos performed by females

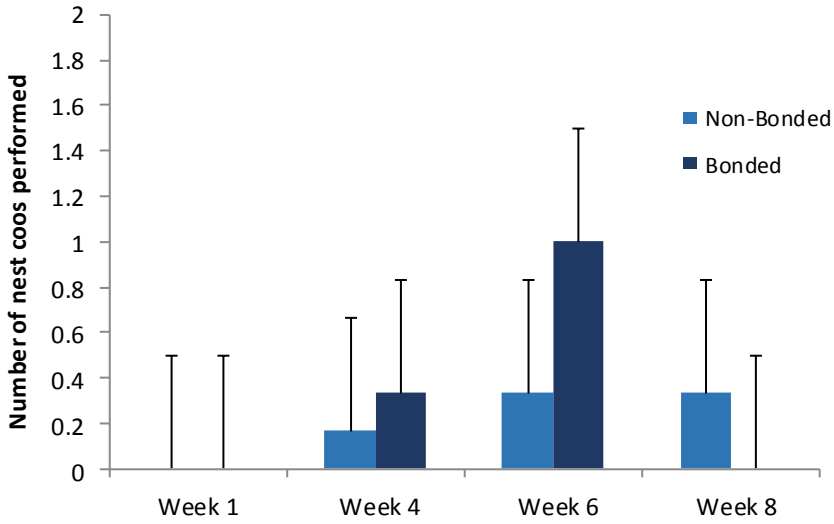


Figure 11: Amount of nest coo behavior performed by novel females in bonded and non-bonded groups

Preference tests done at the eight week recovery time point indicate that males in the bonded group (M=96.5, SD=5.2) preferred to spend a greater percentage of time with their bonded mate, while those in the non-bonded group (M=53.6, SD=8.9) did not have a preference for time spent with females they were tested with (t (10)=10.11, p=0.001) (See figure 12).

Figure 12: Percentage of time spent with mate

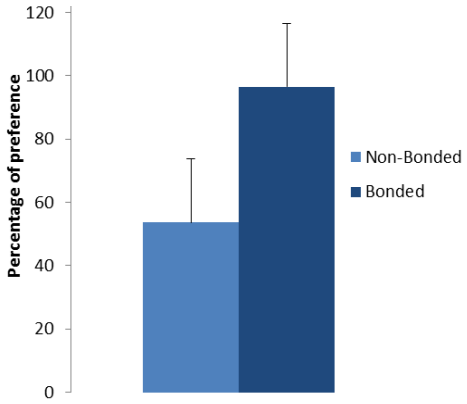


Figure 12: Percentage of time animals preferred to spend with a mate (if in bonded group) or a novel female (if in non-bonded group)

Experiment 4: Effect of destruction of memory of mate, via taeniae lesion, on neurogenesis and courtship behavior during recovery period following VMN lesion.

Pair bonding has been found to moderate courtship (nest coo) behavior (chapter 2) via the nucleus taeniae's large fiber projection to the VMN (Cheng et al 1999). In the previous experiments (1-3), we identified that the male's own nest coo behavior, which engages the VMN circuitry, is a key factor in stimulating neurogenesis in the VMN following lesions. In the following experiment, we assess whether damage to the taeniae in bonded doves, which disrupts a dove's ability to maintain a pair bond, will have an effect on neurogenesis in the VMN following lesions by eliminating bonding's effect on moderating the intensity of courtship behavior.

Methods:

Subjects:

Sixteen male doves that were bred and housed in an AAALAC accredited animal care facility at Newark, Rutgers University were allowed to go to the breeding cycle. The birds were dived into two group: a bilateral double lesion group (taeniae and VMN) and a bilateral single lesion group (VMN). One bird in each group were used for sham lesions to ensure that results were not due to surgical procedure. Birds were housed with their mates.

Electrolytic Lesions to VMN and nucleus taeniae:

VMN lesions followed procedures listed in Chen (2006). Briefly, doves were anesthetized using Chloropent (2.5 ml/kg) before the initiation of surgical lesions. In addition, Marcaine (.0125%) was infiltrated subcutaneously along incision line prior to the lesion. The dove's head was fixed on a sterotaxic instrument at an angle of 45 degrees. A 0.03 mm electrode (O.D. tungsten wire) was inserted into the following coordinates: 2.6 mm anterior to the interaural line (L), 0.5 mm lateral to the midline (DV), and 8.5 mm ventral to the dura. One milliamp of positive current was administered for 30 seconds. Doves were lesioned bilaterally. The double lesion group (taeniae+VMN) had an additional lesion done at the time of the surgery. A 0.03mm electrode (O.D. tungsten wire) was inserted into the following coordinates: 4.5 mm anterior (A), 4.5 lateral, and 5.5(L), and 5.5mm ventral (V), in accordance with previous lesion studies done in our laboratory (Cheng 1992). One milliamps of positive current was administered for 30 seconds for each of the bilateral lesions. Doves were sutured following the procedure and allowed to recover. Sham lesioned birds underwent the same procedure, but no current was administered at the time of the surgery. BrdU injections were done in accordance to procedures in experiment 1.

Behavioral Observation:

Males were housed in isolation a day pre-testing and were subjected to a similar type of test as they were in experiment one. During test day, males were put into cages with opaque dividers with their mated female, the dividers were lifted and behavior was observed for one hour, as detailed above. In addition, female behavior was recorded during the testing session to determine amount of courtship behavior produced by the

stimulus. Wing flips, cackles, preening, nest coo, perch coos were recorded during this allotted time frame. Behavioral observations for lesioned pair bonded birds and their stimuli were done at a baseline (pre-lesion), 1 week post-lesion, 4 weeks post-lesion, 6 weeks post-lesion, and 8 weeks post-lesion time frame. Other types of behaviors including pecking, eating, walking, and drinking were also measured. A preference test (described in experiment one) was conducted at week 8 to determine if doves retained a preference (attachment) for their pair bonded mate. Doves were sacrificed an hour following the preference test. Brains were extracted, immunolabeled, and counted in accordance to procedures listed in experiment one.

Results:

We ran a glm assessing differences in the amount of double labeled cells (NeuN+BrdU) in the VMN (outcome variable) with type of lesion (VMN vs VMN+taeniae). We used amount of nest coo behavior during weeks 1, 4, 6, and 8 as a covariates. Nest coo data was transformed using a ln(x+1) to correct for differences in variance across groups while allowing for the maintenance of "0" value data (Fletcher et al 2005). Our model found that there was an effect of lesion type on number of double labelled cells ($F(7,48)=69.6$, $p=0.000$). There was an effect of the male's own courtship behavior ($F(7,48)=2.75$, $p=0.05$). There was no effect on week the behavior was performed ($F(7,48)=0.725$, $p=0.54$.

Not taking into account other factors, double labeled cell counts in the VMN at the 8 weeks recovery point after lesion, were significantly higher ($t(12)=4.612$, $p=0.0003$) in doves that had a VMN/TN double lesion ($M=32.68$, $SD=7.32$) than those that had a

VMN lesion alone (M=12.5; SD=8.9). (See figure 13). Nest coo behavior at 8 weeks indicates that VMN/TN double lesioned doves (M=200.3, SD=129.3) displayed a higher amount of nest coo (courtship) behavior (t(12)=1.97, p=0.036) towards females than doves that were VMN single lesioned (M=90.6, SD=70.35). Males in the double lesioned group (week 4: M=243.6, SD=155; week 6: M=174.3, SD=104) also displayed higher rates of nest coo behavior at 4 weeks (t(12)=2.5, p=0.01) and 6 weeks(t(12)=2.42, p=0.02) than those in the single lesioned group (week 4: M=81.6, SD=67.2; week 6: M:70.4, SD=44.6). The first week after lesion, doves in the double lesion (M=0.57; SD=0.78) and single lesion group (M=0.571; SD=1.512) showed no difference in behavior (t(12)=0; p=0.5) (See figure 14).

Figure 13: Number of double labeled BrdU/NeuN cells

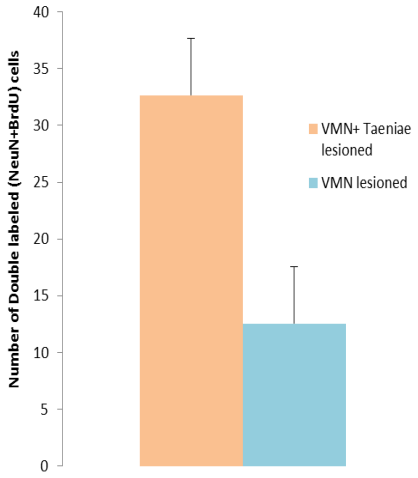


Figure 13: Number of double labeled (BrdU+NeuN) cells in VMN of doves with VMN single lesions and VMN+taeniae double lesions

Figure 14: Number of nest coos performed

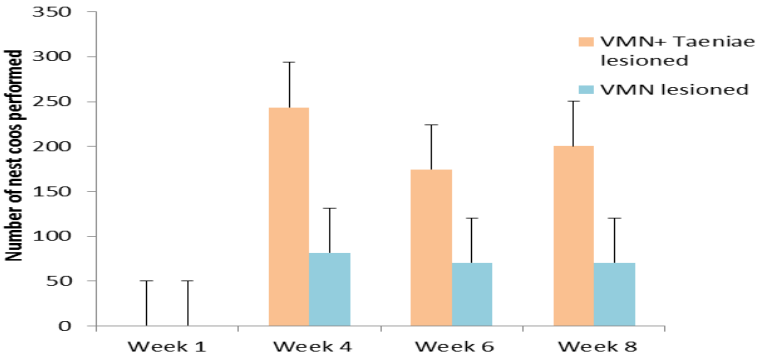


Figure 14: Number of nest coos performed during recovery period following VMN lesion in doves with VMN single lesions and VMN+taeniae double lesions

Preference tests at 8 weeks indicate that males that were VMN single lesioned (M= 81, SD=13.8) preferred to spend more time (t (12)=2.47;p= 0.015) with their mate than those that were VMN/TN double lesioned (M=52.7, SD=24) (See figure 15).

Figure 15: Percentage of time spent with mate

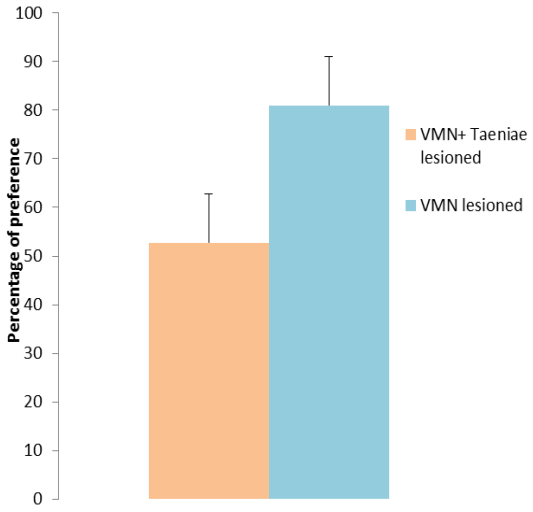


Figure 15: Percentage of preference for mate at 8 weeks recovery period following VMN single lesions and VMN+taeniae double lesions

Statistical Analysis using data from all experiments (Experiments 1-4):

Factors that influenced double labeled cell counts (NeuN/BrdD) and courtship behavior during the 8 week recovery period were analyzed using data from all experiments with general linear mixed-effects model (glmm). For double labeled cell counts we ran a model using data from experiments 1, 3, and 4, since we did not gather cell count data from experiment 2. Data from all experiments was included for analysis of factors that include courtship behavior. We included experiment type as a random effect to adjust for variance due to the data being gathered during different experiments. Specifically, we assessed whether lesion type (have a double-lesion (taeniae+VMN) versus single lesion (VMN), housing condition (being housed with a mate versus being housed with a novel female), or animal used as stimulus during preference test affected number of double labeled cells and courtship behavior at the 8 week recovery point. Analysis for individual experiments was also done.

Results from analysis of all experiments (1-4):

Generalized Linear Mixed Effects Model using data from all experiments:

A generalized linear model was used to assess whether lesion type (VMN or VMN+taeniae), bond status, and testing stimulus (whether birds was tested with mate versus a stimulus) had an effect on number of double labeled (NeuN/BrdU) after eight weeks of recovery. Data was taken from experiments 1, 3, and 4 since cell counts were not analyzed in experiment 2. A log link function was used in order to account for differences in variance between groups. While bond status (X^2 (1,N=38)=6.772,

p=0.009) had an effect on the number of double labeled cells, no effect was seen for experiment number (X^2 (2, N=38)=2.669, p=0.263). A marginal effect was found for whether the taeniae was lesioned in conjunction with the VMN (X^2(1,N=38)=3.106, p=0.078).

Discussion:

Our current study tested whether bond status, a social factor that alters the valance of a female stimulus for the male she is mated to, has an effect on neurogenesis and recovery of function after damage to the VMN, a nucleus involved with regulation of hormones for reproductive behaviors. In modeling data from all experiments, we found that doves in the non-bonded group had a significantly higher number of double labeled (NeuN/BrdU) cells, a marker of a differentiated, mature new neuron (X^2(1,N=38)=6.772, p=0.009). These results held true regardless of female stimuli (mate versus novel stimulus) used during behavioral testing (experiments 1-3). During the recovery period following VMN lesions, males in the non-bonded group had higher amounts of courtship behavior than those in the bonded group (F (7,40)=4.38, p=0.43), regardless of the amount of behavior the female stimuli performed. This is consistent with our previous study (chapter 2), that show that doves that have not formed a bond show greater amount of courtship (nest coo) behavior than those that are bonded. VMN lesions, therefore, did not seem to disrupt bonding in ring doves. Results of preference tests done during the eight week recovery time point confirm this result. Following damage to the VMN, males in the bonded group preferred to spend time with their mates during preference tests indicating that the male's ability to bond with his mate was not affected.

Damage to the nucleus taeniae (avian amygdala) prevents doves from choosing to spend time with their mate during laboratory preference tests and from preferentially performing nest coo behavior towards females that they are bonded to, but does not prevent doves from engaging in courtship behavior (chapter 2). Once bonded, ring doves bypass portions of the courtship routine in order to progress to later stages of the breeding cycle more quickly. This results in bonded males producing less amounts of nest coo behavior than doves that have not formed bond. Lesions to the nucleus taeniae eliminate the memory of a mate with whom they completed the breeding cycle with which disrupts pair bonding's effect on reproductive behavior towards the mate, increasing its intensity during the courtship (nest coo) phase. While ZENK studies suggest that the anterior medial region of the taeniae is responsible for regulating bonding, we lesioned the entire taeniae during this experiment. Full taeniae lesions allow for other emotional behaviors, such as fear, to influence our results (Cheng et al 1999). The results of our current study show that bilateral lesions to the taeniae disrupts the effects of bonding on VMN induced neurogenesis and recovery of courtship behavior following VMN lesions. Double (VMN+taeniae) lesioned doves produce a higher amount of courtship behavior during behavioral tests at 4 ($t(12)=2.5$, $p=0.01$), 6 ($t(12)=2.5$, $p=0.01$), and 8 ($t(12)=1.97$, $p=0.036$) weeks, consistent with nest coo levels produced by doves that are not bypassing early portions of the breeding cycle. Consistent with behavioral data, bonded doves with double (VMN+taeniae) lesions have a greater amount of double labeled BrdU/NeuN cells at the eight week recovery period than intact bonded doves ($t(12)=4.612$, $p=0.0003$). Preference tests at the 8 week recovery time point found that doves in the taeniae

lesioned group did not choose to spend more time with their mates than a novel female, while doves in the intact group had a preference, affirming that pair bonding status can modulate circuitry responsible for courtship behavior. Other behaviors, such as walking, eating, etc., produced by the male were also recorded. While there were differences between groups in the number of other behaviors produced by males, these differences were not consistent. For example, males in the bonded group displayed significantly higher numbers of other behavior during week 6 behavioral tests, but fewer amounts during week 8. Nest coo behavior once recovered (week 4) , however, remained consistent across weeks.

Could intensity of female behavior cause variation in the male's courtship output in the present study? Observations of the female stimuli's behavior during behavior tests suggest that female behavior did not directly influence group differences in the male's courtship behavior. Female stimuli did not differ in the amount of nest coo behavior performed toward the male, regardless of which group housing condition they were in, with the exception of the fourth week behavioral test (experiment 2), in which the female in the bonded group produced more amounts of behavior than the non-bonded group. During our third experiment, when males were uniformly tested with a novel female, regardless of housing conditions, males in the non-bonded group continued to display higher amounts of nest coo behavior and had greater numbers of double labeled cells (NeuN/BrdU) than those in the bonded group. This suggests that it is the male's own courtship behavior, and not the perceptual input of the female's behavior or other types of

behavior performed by the male that is stimulating greater numbers of double labeled cells (NeuN/BrdU) and greater behavioral (nest coo) recovery in the VMN after lesions.

Although we have shown that the male's own behavior aids in the production of new neurons, this data alone cannot eliminate the possibility that the behavior performed by the female stimulus had some role in this outcome. Our study was unique in that it allowed for the males to be tested with stimuli that were visually similar, but that had their own unique social contexts which promoted varying amounts of courtship behavior from the males. Our results suggest that the female's behavior was not driving the male's nest coo performance or the difference in the production of new neurons between males that were exposed to bonded (familiar) females and to non-bonded (unfamiliar) females. However, novelty may have played a role in the way in which the male responded, namely, the male in the non-bonded group courted more rigorously than that in the bonded group. We conclude, therefore, that while it was the male's own behavior that was involved in this rate difference, it was mediated by the stimulus property. This result is consistent with the hypothesis that engagement of the circuitry lesioned (in this case, circuitry mediating nest-coo behavior) facilitates the consolidation of new born neurons in this region (VMN) (Cheng 2013). In both males and females, pair bonding is formed only after the doves performed a complete sequence of the breeding cycle (Morris and Erickson 1971) or when they perform behavior (nest coo) specifically linked to successful breeding (experiment 3). It thus appears that the visual properties of the stimulus that invoke a different level of male response are not merely perceptual

properties (familiar versus non-familiar), but the specific value attached to the properties, the bonding, in our case.

Despite evidence that activity mediated by neurogenic areas can aid in the increase of new neuron in these regions, some tasks are thought to be mediated by a similar circuitry response, specifically hippocampal mediated behaviors, do not alter new neurons in this neurogenic region (Shors et al 2002). There are several possible reasons why this might occur. Some tasks may engage the circuitry at a higher level than other tasks. The behavior's influence on the circuitry may also be threshold, time, or stage dependent. Behavior not performed at a high enough threshold or at the wrong time period or stage may not produce expected outcomes. Differences in results produced by studies measuring the same task have been reported (Gould et al 1999;van Pragg et al 1999). In the case of these studies, animals that were allowed a longer exposure time to the task produced a greater amount of new neurons in the area of interest. A key feature of our study is that we used an electron confocal microscope that allowed us to assess samples in three dimensional space, allowing us to confirm that every single cell that was counted as a differentiated new neuron had markers that were colocalized in the same cell and that these markers were in the appropriate region of the cell. Many studies do not do this, but instead assess only the production of newly produced cells, which may not develop into mature neurons (Shor et al 2002, van Pragg et al 1999, and Gould et al 1999). While this technique is unused by most studies involving behavior, not analyzing cell counts this way may lead to false positives when assessing double labeled cells and might alter the results of experiments, particularly when comparing rates of neurogenesis across groups.

Our results suggest that levels of increased rate of neurogenesis are influenced by activity that engages the circuitry where the new neurons are being measured. Behavior, however, does not happen in a vacuum. The male doves in our experiment were responding to females they encountered, but the level of courtship was affected by whether the doves were previously pair bonded. Properties of the female stimulus the males were housed with (and if in the bonded group, formed a bond with) modulated the male's behavior, and therefore, are themselves a factor mediating the production of new neurons and behavioral recovery associated with it. In lesion studies in doves (Cheng et al 2004; Chen et al 2006), full behavioral recovery and a sufficient amount of neurogenesis to seal the lesion site was produced following a 6-8 week recovery period following surgery and being housed with a mate. In the present study, no courtship (nest coo) behavior was performed during week one post lesion. In experiment two, there was a spike in the nest coo behavior at four weeks for the males exposed to unfamiliar females. The observation that male behavior, in fact, rises at week four (experiment 2) fosters an interesting question. If male's level of courtship is directly related to level of neurogenesis, one would expect to see a higher level of neurogenesis in males following optimal female stimulation (stimulation by novel females) at week four rather than week eight, as once thought. It should be noted that the 8 week recovery timeline was based on male doves that were housed with their mate and not with an unfamiliar female (Chen et al 2006). Quantification of double labeled NeuN/BrdU cells at week four will verify the validity of our interpretation.

General Discussion**:**

Pair bonding is an exclusive mating relationship between two animals that has arisen in a subset of species to aid in survival of the animal or its ability to produce offspring. While bonding is based on attachment (preference) to an animal's mate, it appears that the types of behaviors that initiate a pair bond or that are influenced by bonding differ across species. Attachment is an abstract concept, but can be measured by preference or proximity to the object the animal is attached to (Wickler 1975). The aim of this thesis was to determine which brain regions were responsible for the formation of attachment to one's mate and how this attachment is represented in the brain, allowing animals to remember who their mate is and, thereby, enabling the maintenance of the bond. Our approach was to first determine whether pair bonding was encoded in the brain and how it influences the individual's behavior towards a bonded mate. Secondly, we wanted to identify neural substrates regulate pair bonding. Thirdly, we wanted to determine what factors lead to the formation of pair bonding.

Our first study shows that pair bonding is represented in a region of the brain: the nucleus taeniae. We found that doves which went through a breeding cycle and, were therefore bonded to their mate (Morris and Erickson 1971), had significantly more ZENK stained cells in the anterior portion of the nucleus taeniae (avian amygdala) than those that were not pair bonded. Using a classifier analysis, ZENK stained cells in this area were able to predict whether doves were bonded with high accuracy (100% and 94%) (Chapter 1). Immediate early gene ZENK is a transcription factor that is expressed when the brain responds to external stimuli relating to reproductive behavior. Zebra finch that

performed reproductive behaviors, clumping and preening, had greater amounts of positively labeled ZENK cells than those that did not display these behaviors (Svec et al 2009). While doves generally prefer to spend time in proximity to their mate (Morris and Erickson 1971; Clavijo and Cheng 2007) during laboratory preference tests, there are instances when this is not the case, consistent with behaviors seen in pair bonded birds in the wild where straying is not uncommon. Preference tests are subject to experimental and procedural factors that could impact the result of the test. While we aim to control for these factors, it may not always be possible to do so. The ZENK marker for bonding, however, appears to be a reliable method for which to measure pair bonding.

The nucleus taeniae's high predictability of ZENK expression in identifying bonded mates suggests that this brain region is encoding pair bonding. This neural representation of an emotional memory of the pair bonded mate could also be a neural substrate for the maintenance of the bond. We followed up with a lesion study to address this issue. We found that doves that were lesioned in this area after bonding did not retain a preference for their mate. Additionally, these doves seemed to display higher numbers of nest coo (courtship) behaviors than doves that were not taeniae lesioned. It is important to note that our lesion site was not limited to the anterior portion of the nucleus taeniae ,which has been found to mediate bonding. By lesioning the entire taeniae, other known functions of the taeniae, including the mediation of fear response, can also be disinhibited. In non-lesioned doves, those that were pair bonded had lower amounts of nest coo behavior than those that were not bonded. Pair bonding, therefore, moderates a dove's courtship behavior presumably through the nucleus taeniae's fiber connection to

the VMN (Cheng et al 1999). Neural connectivity between the taeniae and the VMN would suggest that the taeniae may exert a commanding effect of VMN mediated courtship behavior. To test whether the nucleus taeniae mediates pair bonding and the execution of courtship behavior we assessed whether doves of different bonding statuses (pair bonded versus not pair bonded) had differences in courtship behavior following bilateral taeniae lesions. Following taeniae lesions, we found that doves that were pair bonded lost preference for their mate, but did not have differences in courtship behavior to those in the non-bonded group. This finding suggests that taeniae lesions disrupt pair bonding, causing doves to court indiscriminately towards doves they are bonded to and not bonded to; it, however, did not disrupt ability to court. Similarly, lesions to the amygdala in prairie voles erase mate preference and allow for engagement in pre-bonding behaviors (Demas et al 1997), suggesting that the amygdala's (taeniae) mediation of bonding may be conserved across species.

Courtship behavior (nest coo) is regulated by the ventromedial nucleus of the hypothalamus (VMN). Doves with VMN lesions lose their ability to perform nest coo behavior (Bernstein and Cheng 1992). Following VMN lesions, newborn cells from the subventricular zone migrate to the VMN and mature (Cao and Cheng 2002). Under the right conditions, doves will recover courtship behavior after a period of eight weeks. Chen et al found that optimal housing conditions facilitate the differentiation process of newborn neurons, for example stimulus females that invoke VMN dependent male courtship behavior (nest coo), affecting the amount of double labeled NeuN+BrdU cells in the VMN (2006). Since courtship behavior is moderated by the formation of a pair

bond, we assessed whether bond status would affect neurogenesis and recovery of courtship function after lesions to the VMN in chapter 5. We tested male doves that were not bonded and housed with a rotation of novel females and those that were bonded and housed with their bonded mate to determine whether there would be differences in double labeled NeuN+BrdU cells and recovery of courtship behavior after VMN lesion in these groups. Male doves in the non-bonded group had greater numbers of NeuN+BrdU double labeled cells after eight weeks of recovery than those in the bonded group. Male courtship (nest coo) behavior in the non-bonded group was also higher in this group across several weeks during the recovery period. Being housed with a novel female, therefore, increased reinstatement of lost cells and function. To determine whether higher amounts of the male's nest coo could be accounted for by greater amounts of female nest coo from which the male was subjected to, we assessed both the male and female's behavior during the recovery period following VMN lesion in a subsequent experiment. If a female's courtship (nest coo) behavior was driving nest coo performance in males, we could expect females in the non-bonded group to display greater amounts of nest coo behavior. We found that females did not have differences in performance of courtship behavior measured, except during week four, when females in the bonded group had higher amounts of nest coo behavior. Regardless, males in the non-bonded group performed greater amounts of nest coo behavior towards non-bonded females than males in the bonded group towards their mate suggesting that the perceptual stimulus property (bonding) makes a difference in the amount of male behavior performed regardless of level of female behavior. To rule out the effects of other behaviors we analyzed other types of behavior such as walking around, pecking, and eating and found that although

these behaviors differed in a males across weeks of testing, which group performed higher amounts of behavior varied across week of testing suggesting there was no pattern that supported that one group of males performed these behaviors at a higher amount than the other.

In the aforementioned experiments, doves were tested with the bird they were housed with during behavior tests. In order to control for whether this had an effect on our results, we re-ran the experiment using a novel stimulus female during the behavior tests. Like in the previous version of this experiment, we found that males in the non-bonded group performed a greater amount of nest coo behavior and had a higher number of double labeled NeuN+BrdU cells than those in the bonded group. This suggests that the behavior level of both the housing and testing stimulus (females) does not have an effect on the intensity of male courtship level which in turn does not affect the rate of neurogenesis. Exposure female stimuli of a different value (female of different bond status, e.g.), however, created differences in the male's behavior. Males that were pair bonded produced less amounts of nest coo behavior towards its mate than those that were not bonded. This study suggests that the male's own nest coo behavior, which is mediated by the VMN, stimulated higher levels of neurogenesis and, in turn, recovery by engaging circuitry specific to its function.

In light of the taeniae's projection to the VMN, we assessed whether bilateral taeniae lesions result in changes in amount of mature new neurons and recovery of courtship behavior in doves. Taeniae based pair bonding is responsible for modulating courtship behavior after bonding by lowering courtship levels once bonded (chapter 2). We found that bonded doves that had dual taeniae/VMN lesions had higher amounts of

nest coo behavior during the eight week recovery period, presumably due to the destruction of the taeniae, which eliminated pair bonding's effect on nest coo levels towards their mates, and had higher amounts of double labeled NeuN+BrdU cells after eight weeks.

Results of the taeniae lesion on pair bond formation (chapter 2) suggest that the nucleus taeniae encodes events leading to the formation of pair bonding, specifically associating the identity of a particular mate with having successfully completed the breeding cycling, which is an assurance for the prediction of breeding success. Our results, however, do not address whether this region specifically encodes pair bonding. In fact, the taeniae may encode bonding unrelated learning memory. To address this possibility, we assessed whether taeniae lesions affected a dove's ability to remember objects that were unrelated to bonding or the memory of their mate. Because the amygdala has been linked with emotional memory, we used stimuli that were not high in emotional salience to the birds, mainly, rubber toys. In order to establish a baseline before taeniae lesions, we exposed doves to one of the objects and allowed doves to go through the preference test to determine whether they had a preference for novel or familiar objects. During pre-trials doves did not show preference for either novel or familiar objects. We conducted pre-trial tests several times with variations in time periods of exposure and doves still showed no difference in choosing objects that were either novel or familiar. We suspect that, because the objects were not relevant to the bird's survival or to caring for their young, objects were not salient enough for the birds to remember and, unlike rodents that rely on tactile cues to find their pups allowing them to perform well on this testing measure , birds may rely on other cues when remembering

objects. Most likely, none of the objects used were remotely relevant to a bird's natural habitat.

The memory of bonding is strikingly displayed when doves show a preference for the partner they went through the cycle with, even after an extended period of separation (Morris and Erickson 1971). Based on this study, it was assumed that a complete breeding cycle was necessary for bond formation. We tested to see if this was the case. The breeding cycle in doves follows a predictable pattern of behavior in successive stages (early courtship, nest-coo phase, nest-building and egg laying phase, and squab rearing phase) (Wallace 1908; Miller and Miller 1958; Lovori and Hutchinson 1975; Lehrman 1964; Lehrman 1959), allowing us to determine when, during the cycle, bonds are formed and which types of behavior patterns may be essential for bond formation. Positively labeled ZENK cells in the nucleus taeniae, which reliably predict bond formation, the memory of the specific animal with whom the dove went through the bonding cycle (chapter 1), were analyzed to determine when during the breeding cycle bonding is formed. Male and female doves were tested across multiple stages during the breeding cycle including the bow coo (greeting) stage, nest coo (courtship) stage, egg laying stage, squab rearing stage, and completion of the entire cycle and comparing them to doves that had not gone through the breeding cycle. We found that in both males and females, doves that had gone through the nest coo behavior, egg laying behavior, squab rearing behavior, and full cycle significantly differed in number of positively labeled ZENK cells in the nucleus taeniae from those that had gone through the bow coo phase or had not gone through the breeding cycle at all. When assessing mean differences in amounts of ZENK labeled cells between doves that had not undergone the breeding cycle and those that had

gone through the stages described above, we found differences in all but the bow coo stage in both males and females. The pair bond, therefore seems to become registered during the nest coo stage of the breeding cycle. It is difficult to determine whether nest coo behavior itself or the combination of nest coo and bow coo behavior are necessary to form the bond. Because the breeding cycle happens in a predictable sequence and nest coo behavior cannot exist without first performing bow coo, the initiation of pair bonding could occur due to do the additive effect of nest coo phase and bow coo phase. Females, however, do not bow coo, so this presumably does not apply to females. Completing the nest coo behavior stage, however, seems to be essential to pair bonding. Doves that bond exhibit changes in the amount of nest coo behavior performed towards their mate and damage to the nucleus taeniae not only alters preference for a mate, a measure of bonding behavior, but also bond related levels of nest coo behavior towards their mate, as seen in the second breeding cycle. Since bow coo alone is not sufficient to form a bond and the duration of time associated with exposure to dove does not alone lead to bond formation, execution of nest coo behavior plays a key role in bond formation. Importantly, Cheng et al documented electrophysiological evidence that female nest coo triggers a female's own reproductive-endocrine system culminating in fertilization and egg laying and leading to a successful cycle (1998). Our conclusion is, therefore, in line with the probable evolutionary motives of pair bonding- successful breeding.

In chapter 4 we addressed whether the nucleus accumbens, an area affiliated with pair bonding in prairie voles, a widely cited animal model for pair bonding in mammals, is also involved in mediating the formation or maintenance of pair bonding in ring doves. We tested males that were already bonded and those in a non-bonded group to determine

if lesions to the nucleus accumbens altered preference for mate or amount of nest coo behavior performed. We found that bilateral lesions in this region did not affect the amount of nest coo, bow coo, or preference for mate (or a novel female in the non-bonded group) in bonded and non-bonded doves, suggesting that they did not have an effect on bond status. Lesions to the nucleus accumbens did not prevent doves from going through the breeding cycle and forming a bond. The nucleus accumbens, therefore, does not appear to mediate bonding behaviors in ring doves in the same way that it does in prairie voles. In prairie voles, bonding is formed after 24 hours of cohabitation or, if mating occurs, during the first six hours after being paired (Williams et al 1992). Although prairie voles engage in nasogenital grooming, a behavior which primes the female for mating, voles do not have extensive courtship periods. Bonds are also broken fairly quickly in voles. If males are separated from females from a period of 24 hours, females will be ready to remate (Carter et al 1986). While no study has assessed which behaviors are necessary for pair bonding in prairie voles, it seems that pre-copulatory behavior (nasogenital grooming) may be central to bonding in voles. Copulation is mediated by the hypothalamus in birds and mammals. Completion of a sexual act activates the rewarding properties of this behavior. Involvement of the nucleus accumbens, a region responsible for the hedonic component of sexual behavior, therefore is no surprising. The nucleus accumbens, while not found to mediate pair bonding in doves, has been implicated in the regulation of sexual behaviors in birds (Tlemcani et al 2000; Husband 2004). This suggests that in birds, as well as in mammals, the rewards system is involved in processing cues associated with sexual behavior. We do not have evidence that this is the case in doves. Doves that had damage to their nucleus accumbens

still participated in sexual behaviors, as expected since the nucleus accumbens does not function in initiating sexual behaviors. Mounting behavior was performed in very small amounts during behavior tests and spot checks making it difficult to compare sexual behavior in lesioned and non-lesioned groups, therefore it remains possible that the nucleus accumbens has an effect on sexual behavior in doves.

Pair bonding has been found to occur across a variety of species. While there is no known phylogenetic pattern that establishes which species pair bond, animals that bond have one trait in common: an attachment to their mate. Attachment to one's mate requires that an animal remembers who their mate is and prefers them to other animals they may encounter. Pair bonding, however, also requires that this attachment be returned by the mate. In doves, bond formation requires a period of courtship (nest coo) behavior (chapter 3), inherent in these behaviors is an interactive, coordinated sequence between a male and female of a pair that advances the breeding stage (Cheng 2011). This testifies to the intimate relationship of the pair which is the key to forming a bond. Pair bonding has been studied, but a distinction has not been made between behaviors that function in forming a pair bond and behaviors that occur following bond formation. We found that behaviors that lead to bond formation and those that follow it can be distinguished. In many species, including ring doves, reproductive and courtship behaviors can occur in the absence of a bond. Bonding has an effect on these behaviors allowing them to bypass introductory courtship levels towards their mate. The area found to mediate bonding in doves, the nucleus taeniae (avian amygdala), has been found to be an accurate marker of preference. Emotional memory for a mate that leads to preference in prairie voles, the most commonly used model for studying bonding, has been found to be mediated by the

amygdala (Demas et al 1997). Although, evidence points to the amygdala/nucleus taeniae being a neural substrate that regulates preference in both of these species, the study assessing the amygdala's role in bonding in voles is not well cited. Rather, the more popularly cited studies assessing neural substrates that regulate bonding in this species focus on the nucleus accumbens, an area found to regulate the blocking of aggressive behavior in bonded voles which occurs following the bond. Since the nucleus accumbens is known to be involved in hedonic aspects of sexual behavior, its role in bonding in prairie voles is not surprising. While other neural substrates may be involved in bonding in other species, the amygdala seems to mediate this in both mammals (voles) and birds (ring doves). This thesis serves as a reminder of the importance of studying mechanisms of complex behavior in multiple species when assessing evolutionary behavior. While we believe that the amygdala may serve as a common neural substrate in regulating attachment between pair bonded animals, more species must be studied to determine whether the amygdala's involvement in retention of bonding is conserved across animals that form bonds.

Bibliography

Absil, P., Branquenier, J.B., Balthazart, J., Ball, G.F. (2002). Effects of lesions of nucleus taeniae on appetitive and consummatory aspects of male sexual behavior in Japanese quail. *Brain, Behavior and Evolution, 60*(1), 13-35.

Amaral, D. G., Price, J. L., Pitkanen, A., Carmichael, S. T. (1992). Anatomical organization of the primate amygdaloid complex. In J. P. Aggleton (Ed.), *The amygdala: Neurobiological aspects of emotion, memory, and mental dysfunction* (pp. 1-66). New York: Wiley-Liss.

Anderson, R.G., Moskat, C., Ban, M., Grim, T., Cassey, P., Hauber, M. (2009). Egg eviction imposes a recoverable cost of virulence in chicks of a brood parasite. *PLoS One, 4*(11), e7725.

Aragona, B.J., Liu, Y., Curtis, J.T., Stephan, F.K., Wang, Z. (2003). A critical role for nucleus accumbens dopamine in partner-preference formation in male prairie voles. *Journal of Neuroscience, 23*(8), 3483-3490.

Aragona, B.J., Liu, Y., Yu, Y.J., Curtis, J.T., Detwiler, J.M., Insel, T.R., Wang, Z. (2006). Nucleus accumbens dopamine differentially mediates the formation and maintenance of monogamous pair bonds. *Nature Neuroscience, 9*(1), 133-139.

Aragona, B.J., Wang, Z. (2004). The prairie vole (*Microtus ochrogaster*): An animal model for behavioral neuroendocrine research on pair bonding. *ILAR Journal, 45*(1), 35-45.

Bales, K.L., van Westerhuyzen, J.A., Lewis-Reese, A.D., Grotte, N.D., Lanter, J.A., Carter, C.S. (2007). Oxytocin has dose-dependent developmental effects on pair-bonding and alloparental care in female prairie voles. *Hormones and Behavior, 52*, 274–279.

Barash, D.P, Lipton, J.E. (2001). *The myth of monogamy: Fidelity and infidelity in animals and people*. New York, New York: Henry Holt and Company.

Barfield, R. J. (1971). Gonadotrophic hormone secretion in the female ring dove in response to visual and auditory stimulation by the male. *Journal of Endocrinology, 49*, 305-310.

Baxter, M.G., Murray, E.A. (2002). The amygdala and reward. *Nature Reviews Neuroscience, 3*(7), 563-573.

Beecher, M.D., Beecher, I.M. (1979). Sociobiology of bank swallows: Reproductive strategy of the male. *Science, 205*, 1282-1284.

Bernstein, P., Zuo, M., & Cheng, M. F. (1993). Social condition affects the courtship behavior of male ring doves with posterior medial hypothalamic lesions. *Behavioral and Neural Biology, 592*, 120-125.

Birkhead, T.R., Burke, T., Zann, R.,Hunter, F.M., Kurpa, A.P. (1990). Extra-pair paternity and intraspecific brood parasitism in wild zebra finches Taeniopygia guttata, revealed by DNA fingerprinting. *Behavioral Ecology and Sociobiology, 27*(5), 315-324.

Bradley, M.M., Greenwald, M.K., Petry, M.C., Lang, P.J. (1992). Remembering pictures: Pleasure and arousal in memory. *Journal of Experimental Psychology: Learning Memory and Cognition, 8*(2), 379-390.

Brito, I., Britto, L.R.G., Ferrari, E.A.M. (2011). Induction of zenk protein expression within the nucleus taeniae of the amygdala of pigeons following tone and shock stimulation. *Brazilian Journal of Medical and Biological Research, 44*(8), 762-766.

Burns-Cusato, M., Cusato, B.M., Daniel, A. (2005). A new model for sexual conditioning: The ring dove (*Streptopelia risoria*). *Journal of Comparative Psychology, 119*(1), 111-116.

Cahill,L., Babinsky, R., Markowitsch, H.J., McGaugh, J. (1995). The amygdala and emotional memory. *Nature, 377*(6547), 295-296.

Cao, J., Wenberg, K., Cheng, M.F. (2002). Lesion induced new neuron incorporation in the adult hypothalamus of the hypothalamus of the avian brain. *Brain Research, 943*, 90-92.

Carter, C.S., Getz, L.L., Cohen-Parsons, M. (1986). Relationships between social organization and behavioral endocrinology in a monogamous mammal. *Advances in the Study of Behavior, 16*, 109-145.

Chapais, B. (2008). The evolutionary history of pair bonding. *Primeval kinship: How pair-bonding gave birth to human society* (pp. 157-179) Harvard University Press.

Chapais, B. (2011). The evolutionary history of pair-bonding and parental collaboration. In C. Salmon, & T. Shackelford (Eds.), *The oxford handbook of evolutionary family psychology.* (pp. 33-50). New York: Oxford University Press.

Chen, G., Bonder, E., Cheng, M.F. (2006). Lesion induced neurogenesis in the hypothalamus is involved in behavioral recovery in adult ring doves. *Journal of Neurobiology, 66*, 537-551.

Cheng, M. F. and Burke, W. H. (1983). Serum prolactin levels and crop-sac development in ring doves during a breeding cycle. *Hormones and Behavior, 17*(1), 54–65.

Cheng, M.F, Klint, T. Johnson, A.L. (1986). Breeding experience modulating androgen dependent courtship behavior in male ring doves (*Streptopelia risoria*). *Physiology & Behavior, 36*(4), 625-630.

Cheng, M. F. (1973). Effect of estrogen on behavior of ovariectomized ring doves (Streptopelia risoria). *Journal of Comparative and Physiological Psychology, 82*(2), 234-239.

Cheng, M. F. (1977). Role of gonadotrophin releasing hormones in the reproductive behaviour of female ring doves (*Streptopelia risoria*). *Journal of Endocrinology, 74*, 37-45.

Cheng, M. F. (1979). Progress and prospects in ring dove research: A personal view. *Advances in the Study of Behavior, 9*, 97-129.

Cheng, M. F. (1986). Female cooing promotes ovarian development in ring doves. *Physiology and Behavior, 37*(2), 371-374.

Cheng, M. F. (1992). For whom does the female dove coo? a case for the role of vocal self-stimulation. *Animal Behaviour, 43*(6), 1035-1044.

Cheng, M. F. (2008). The role of vocal self-stimulation in female response to males: Implications for state-reading. *Hormones and Behavior, 53*(1), 1-10.

Cheng, M. F. (2011). Newborn GnRH neurons in the adult forebrain of the ring dove. *Hormones and Behavior, 60*, 94-104.

Cheng, M. F. (2013). Hypothalamic neurogenesis in the adult brain. *Frontiners in Neuroendocrinology, 34*(3), 167–178.

Cheng, M.F., Balthazart, J. (1982). The role of nest-building activity on gonadotrophin secretions and the reproductive success of ring doves (*Streptopelia risoria*). *Journal of Comparative and Physiological Psychology, 96*(2), 307-324.

Cheng, M.F., Chaiken, M., Zuo, M., Miller, H. (1999). Nucleus taenia of the amygdala of birds: Anatomical and functional studies in ring doves (*Streptopelia risoria*) and european starlings (*Sturnus vulgaris*). *Brain, Behavior and Evolution, 53*(5-6), 243-270.

Cheng, M.F., Desiderio, C., Havens, M., Johnson, A. (1988). Behavioral stimulation of ovarian growth. *Hormones and Behavior, 22*(3), 388-401.

Cheng, M.F., Havens, M.H. (1993). Auditory-responsive units in the midbrain vocal nuclei in the ring dove (*Streptopelia risoria*). *Brain Research Bulletin, 30*(5-6), 711-715.

Cheng, M.F., Peng, J.P., Chen, G., Gardner, J.P., Bonder, E. (2004). Functional restoration of acoustic units and adult-generated neurons after hypothalamic lesion. *Journal of Neurobiology, 60*, 197-213.

Cheng, M.-F., Peng, J.P., Johnson, P. (1998). GnRH neurons preferentially respond to female nest coo stimulation: Demonstration of direct acoustic stimulation of luteinizing hormone release. *Journal of Neuroscience, 18*, 5477-5489.

Cheng, M.F., Porter, M., Ball, G. (1981). Do ring doves copulate more than necessary for fertilization? *Physiology and Behavior, 27*(4), 659-662.

Cheng, M.F., Silver, R. (1975). Estrogen-progesterone regulation of nest-building and incubation behavior in ovariectomized ring doves (*Streptopelia risoria*). *Journal of Comparative and Physiological Psychology, 88*(1), 256-263.

Cheng, M.F., Zuo, M. (1994). Proposed pathways for vocal self-stimulation: Met-enkephalinergic projections linking the midbrain vocal nucleus, auditory-responsive thalamic regions and neurosecretory hypothalamus. *Journal of Neurobiology, 25*(4), 361-379.

Cherkas, L.F., Oelsner, E.C., Mak, Y.T., Valdes, A., Spector, T.D. (2004). Genetic influences on female infidelity and number of sexual partners in humans: A linkage

and association study of the role of vasopressin receptor gene *(AVPR1A*). *Twin Research, 7*(6), 649-658.

Clavijo, A.,Cheng, M.F. (2007). The role of hypothalamus in pair bonding. *Annual Biomedical Research Conference for Minority Students,* Austin, TX.

Cohen, J., Cheng, M.F. (1979). Role of vocalizations in the reproductive cycle of ring doves (*Streptopelia risoria*): Effects of hypoglossal nerve section on the reproductive behavior and physiology of the female. *Hormones and Behavior, 13*(2), 113-127.

Cohen, J., Cheng, M.F. (1981). The role of the midbrain in courtship behavior of the female ring dove (*Streptopelia risoria*): Evidence from radiofrequency lesion and hormone implant studies. *Brain Research, 207*(2), 279-301.

Crawley, J. N. (1984). Evaluation of a proposed hamster separation model of depression. *Psychiatry Research, 11*, 35-47.

Davies, S. J. J. F. (1974). Studies of the three coo-calls of the male barbary dove. *Emu, 1*(18-26)

Davis, M., Whalen, P.J. (2001). The amygdala: Vigilance and emotion. *Molecular Psychiatry, 6*(1), 13-34.

del-Barco-Trillo, J., Gulewicz, K., Johnston, R.E. (2009). Medial amygdala involvement in discrimination of same-species and closely-related-species male stimuli in estrous female *Mesocricetus* hamsters. *Behavioral Neuroscience, 123*(4), 758-763.

Demas, G.E., Williams, J.M., Nelson, R.J. (1997). Amygdala but not hippocampal lesions impair olfactory memory for mate in prairie voles (*Microtus ochrogaster*). *American Journal of Physiology, 273*(5), R1683-R1689.

Dewsbury, D. (1981). Effects of novelty of copulatory behavior: The coolidge effect and related phenomena. *Psychological Bulletin, 89*(3), 464-482.

Dolcos, F., LaBar, K.S., Cabeza, R. (2004). Interaction between the amygdala and the medial temporal lobe memory system predicts better memory for emotional events. *Neuron, 42*(5), 855-863.

Dunn P.O., Robertson R.J., Michaud-Freeman, D., Boag, P.T. (1994). Extra-pair paternity in tree swallows: Why do females mate with more than one male? *Behavioral Ecology and Sociobiology, 45*(4), 273-281.

Durand, S.E., Tepper, J.M., Cheng, M.F. (1992). The shell region of the nucleus ovoidalis: A subdivision of the avian auditory thalamus. *Journal of Comparative Neurology, 323*(4), 495-518.

Erickson, C., Lehrman, D. (1964). Effect of castration of male ring doves upon ovarian activities of females. *Journal of Comparative and Physiological Psychology, 58*(2), 164-166.

Erickson, C. J. (1970). Induction of ovarian activity in female ring doves by androgen treatment of castrated males. *Journal of Comparative and Physiological Psychology, 71*(2), 210-215.

Erickson, C. J. (1986). Social induction of the ovarian response in the female ring dove. *Annals of the New York Academy of Sciences, 474*, 13-20.

Erickson, J. E. (1973). Mate familiarity and the reproductive behavior of ringed turtle doves. *The Auk, 90*(4), 780-795.

Everitt, B.J., Cardinal, R.N., Parkinson, J.A., Robbins, T.W. (2003). Appetitive behavior: Impact of amygdala-dependent mechanisim of emotional learning. *Annals of the New York Academy of Sciences, 985*, 233-250.

Fink, S., Excoffier, L., Heckel, G. (2006). Mammalian monogamy is not controlled by a single gene. *Pnas, 103*(29), 10956-10960.

Fletcher, D., Mackenzie, D., Do Villouta, E. (2005). Modelling skewed data with many zeros: A simple approach combining ordinary and logistic regression. *Enviornmental and Ecological Statistics, 12*, 45-54.

Forstmeier W., Kempenaers B., Meyer A., Leisler B. (2002). A novel song parameter correlates with extra-pair paternity and reflects male longevity. *Pro Bio Sci, 269*(1499), 1479-1485.

Fraley, R. C., Brumbaugh, C. C., & Marks, M. J. (2005). The evolution and function of adult attachment: A comparative and phylogenetic analysis. *Journal of Personality and Social Psychology, 89*(5), 731-746. doi:10.1037/0022-3514.89.5.751

Friedman, M., Lehrman, D.S. (1968). Physiological conditions for the stimulation of prolactin secretion by external stimuli in the male ring dove. *Animal Behaviour, 16*(2-3), 233-237.

Friedman, M. B. (1977). Interactions between visual and vocal courtship stimuli in the neuroendocrine response of female doves. *Journal of Comparative and Physiological Psychology, 91*(6), 1408-1416.

Fusani, L., Hutchison, R.E., Hutchison, J.B. (1997). Vocal-postural co-ordination of a sexually dimorphic display in a monomorphic species: The barbary dove. *Behaviour, 134*(5-6), 321-335.

Gage, F.H., Kempermann, G., Palmer, T.D., Peterson, D.A.,Ray, J. (1998). Multipotent progenitor cells in the adult dentate gyrus. *Journal of Neurobiology, 36*, 249–266.

Gallagher, M., Chiba, A.A. (1996). The amygdala and emotion. *Current Opinion in Neurobiology, 6*(2), 221-227.

Garcia, J.R. , MacKillop,J., Aller, E.L , Merriwether,A.M., Wilson, D.S., Lum, J.K. (2010). Associations between dopamine D4 receptor gene variation with both infidelity and sexual promiscuity. *PLoS One, 5*, e14162.

Gavish, L., Carter, C.S., Getz, L.L. (1983). Male-female interactions in prairie voles. *Animal Behaviour, 31*(2), 511-517.

Getz, L.L., Carter, C. S., Gavish, L. (1981). The mating system of the prairie vole, *Microtus ochrogaster:* field and laboratory evidence for pair-bonding. *Behavioral Ecology and Sociobiology, 8*(3), 189-194.

Gibson, M.J., Cheng, M.F. (1979). Neural mediation of estrogen-dependent courtship behavior in female ring doves. *Journal of Comparative and Physiological Psychology, 93*(5), 855-867.

Gordon H. Orians. (1969). On the evolution of mating systems in birds and mammals. *The American Naturalist, 103*(934), 589-603.

Gould,E., Beylin, A., Tanapat, P., Reeves, A., Shors, T.J. (1999). Learning enhances adult neurogenesis in the hippocampal formation. *Nature Neuroscience, 2*(3), 260-265.

Gowaty, P.A., Bridges, W.C. (1991). Nestbox availability affects extra-pair fertilizations and conspecific nest parasitism in eastern bluebirds, *Sialia sialis*. *Animal Behaviour, 41*(4), 661-675.

Gray, E. M. (1997). Female red-winged blackbirds accrue material benefits from copulating with extra-pair males. *Animal Behaviour, 53*(3), 625-639.

Grelotti, D.J., Klin, A.J., Gauthier, I., Skudlarski, P., Cohen, D.J., Gore, J.C., Volkmar, F.R., Schultz, R.T. (2005). fMRI activation of the fusiform gyrus and amygdala to cartoon characters but not to faces in a boy with autism. *Neuropsychologia, 43*, 373-385.

Griffith, S.C., Owens, I.P., Thuman, K.A. (2002). Extra pair paternity in birds: A review of interspecific variation and adaptive function. *Molecular Ecology, 11*(11), 2195-2212.

Grim, T., Rutila, J., Cassey, P., Hauber, M. (115). Experimentally constrained virulence is costly for common cuckoo chicks. *2009, 1*(14-22)

Hamann, S. (2001). Cognitive and neural mechanisms of emotional memory. *Trends in Cognitive Sciences, 5*(9), 394-400.

Hamann, S., Ely, T.D., Grafton, S.T., Kilts, C.D. (1999). Amygdala activity related to enhanced memory for pleasant and aversive stimuli. *Nature Neuroscience, 2*(3), 289-293.

Hamann, S., Ely, T.D., Hoffman, J.M., Kilts, C.D. (2002). Ecstasy and agony: Activation of the human amygdala in positive and negative emotion. *Psychological Science, 13*(2), 135-141.

Hanson, S. J., Halchenko, Y. O. (2008). Brain reading using full brain support vector machines for object recognition: there is no face-identification area. *Neural Computation*, 20, 486-503.

Hariri, A.R., Tessitore, A., Mattay, V.S., Fera, F., Weinberger, D.R. (2002) The amygdala response to emotional stimuli: a comparison of faces and scenes. *Neuroimage. 17*(1), 317-323.

Harris,V.S.,Sachs, B.D. (1975). Copulatory behavior in male rats following amygdaloid lesions. *Brain Research, 86*(3), 514-518.

Haxby, J.V., Hoffman, E.A., Gobbini, M.I.(2002) Human neural systems for face recognition and social communication. *Society for Biological Psychiatry, 51,* 59-67.

Helmstetter, F.J., Bellgowan, P.S. (1994). Effects of muscimol applied to the basolateral amygdala on acquisition and expression of contextual fear conditioning in rats. *Behavioral Neuroscience, 108*(5), 1005-1009.

Husband, S. A. (2004). *Anatomy and function of the nucleus accumbens in the pigeon (columba livia)* (PhD). Available from scholarcommons.usf.edu.

Hutchison, J. B. (1970). Differential effects of testosterone and oestradiol on male courtship in barbary doves (Streptopelia risoria). *Animal Behaviour, 18*(1), 41-51.

Ikebuchi, M., Hasegawa, T., Bischof, H.J. (2009). Amygdala and socio-sexual behavior in male zebra finches. *Brain, Behavior and Evolution, 74*(4), 250-257.

Ikebuchi, M., Nanbu, S., Okanoya, K., Suzuki, R., Bischof, H.J. (2013). Very early development of nucleus taeniae of the amygdala. *Brain, Behavior and Evolution, 81*(1), 12-26.

Joëls, M., Karst, H., Krugers, H.J., Lucassen, P.J. (2007). Chronic stress: Implications for neuronal morphology, function and neurogenesis. *Frontiners in Neuroendocrinology, 28*(2-3), 72-96.

Karten, H.J., Hodos, W. (1967). *A stereotaxic atlas of the brain of the pigeon: Columba livia.* Baltimore, MD: Johns Hopkins Press.

Kempenaers, B., Verheyen, G.R., Van den Broeck, M., Burke, T., Van Broeckhoven, C., Dhondt, A. (1992). Extra-pair paternity results from female preference for high-quality males in blue tit. *Nature, 357,* 494-496.

Kleiman, D. G. (1977). Monogamy in mammals. *The Quarterly Review of Biology, 52*(1), 39-69.

Klint, T.,Johnson, A. L., Cheng, M-F. (1984). Factors in the dose-response effect of testosterone in the male ring dove. *Physiology and Behavior, 32*(6), 1037-1040.

Komers, P.E., Brotherton, P.N.M. (1997). Female space use is the best predictor of monogamy in mammals. *Proceedings of the Royal Society of London B, 264*(1386), 1261-1270.

Kondo, Y. (1992). Lesions of the medial amygdala produce severe impairment of copulatory behavior in sexually inexperienced male rats. *Physiology and Behavior, 51*(5), 939-943.

Korenbrot, C.C., Schomberg, D.W., and Erickson, C.J. (1974). Radioimmunoassay of plasma estradiol during the breeding cycle of ring doves (Streptopelia risoria). *Endocrinology, 94*(4), 1126-1132.

Krettek, J.E., Price, J.L. (1978). A description of the amygdaloid complex in the rat and cat with observations on intra-amygdaloid axonal connections. *Journal of Comparative Neurology, 178*(2), 255-280.

LaBar, K.S., Cabeza, R. (2006). Cognitive neuroscience of emotional memory. *Nature Neuroscience, 7*(1), 54-64.

Lack, D. (1968). Ecological adaptations for breeding in birds. *Methuen and Co., London,*

Lambe, D.R., Erickson, C.J. (1973). Ovarian activity of female ring doves (*Streptopelia risoria*) exposed to marginal stimuli from males. *Physiological Psychology, 1*(3), 281-283.

LeDoux, J. (2003). The emotional brain, fear, and the amygdala. *Cellular and Molecular Neurobiology, 23*(4-5), 727-738.

Lehrman, D. S. (1958). Induction of broodiness by participation in courtship and nest-building in the ring dove (*Streptopelia risoria*). *Journal of Comparative and Physiological Psychology, 51*, 32-36.

Lehrman, D. S. (1958b). Effect of female sex hormones on incubation behavior in the ring dove (*Streptopelia risoria)*. *Journal of Comparative and Physiological Psychology, 51*(2), 142-145.

Lehrman, D. S. (1959). On the origin of the reproductive behavior cycle in doves. *New York Academy of Science, 21*(8), 682-688.

Lehrman, D. S. (1964). The reproductive behavior of ring doves. *Scientific American, 211*(5), 2-8.

Leonetti, D., & Chabot-Hanowell, B. (2011). The foundation of kinship: Households. *Hum Nat., 22*(1-2), 16-40.

Lois, C.,Alverez-Buylla, A. (1994). Long-distance neuronal migration in the adult mammalian brain. *Science, 264*, 1145–1148.

Long, K., Salbaum, J.M. (1998). Evolutionary conservation of the immediate-early gene ZENK. *Molecular Biology and Evolution, 15*(3), 284-292.

Lovari, S., Hutchison, J.B. (1975). Behavioural transitions in the reproductive cycle of barbary doves S*treptopelia risoria l.*). *Behaviour, 53*(1-2), 126-149.

Lovejoy, C. O. (1981). The origin of man. *Science, 211*(4480), 341-350.

Lovlie, H.,Pizzari, T. (2007). Sex in the morning of in the evening? females adjust daily mating patterns to the intensity of sexual harassment. *American Naturalist, 170*, 1-13.

Lubjuhn T, Strohbach S, Brün J, Gerken T, Epplen JT. (1999). Extra-pair paternity in great tits (Parus major) – a long term study. *Behaviour, 136*(9), 1157-1172.

Lukas, D., Clutton-Brock, T.H. (2013). The evolution of social monogamy in mammals. *Science, 341*(6145), 526-530.

Maren, S. (1999). Long-term potentiation in the amygdala: A mechanism for emotional learning and memory. *Trends in Neurosciences, 22*(12), 561-567.

Maren, S., Fanselow, M.S. (1996). The amygdala and fear conditioning: Has the nut been cracked? *Neuron, 16*(2), 237–240.

Marlowe, F. (2001). Male contribution to diet and female reproductive success among foragers. *Current Anthropology, 42*(5), 755-760.

Marlowe, F. W. (2003). A critical period for provisioning by hadza men: Implications for pair bonding. *Evolution and Human Behavior, 24*(3), 217-229.

Marzluff, J.M., Miyaoka, R., Satoshi, M., Cross, D.J. (2012). Brain imaging reveals neuronal circuitry underlying the crow's perception of human faces. *Pnas, 109*(39), 15912-15917.

McGaugh, J. L. (2000). Memory—a century of consolidation. *Science/aaas, 287*(5451), 248-251.

McGaugh, J.L., Cahill, L., Roozendaal, B. (1996). Involvement of the amygdala in memory storage: Interaction with other brain systems. *Pnas, 93*(24), 13508-13514.

Miller, W.J., Miller, L.S. (1958). Synopsis of behaviour traits of the ring neck dove. *Animal Behaviour, 6*(1-2), 3-8.

Mock D.W, F. M. (1990). Monogamy and long-term pair bonding in vertebrates. *Trends in Ecology & Evolution, 5*, 39–43.

Morris, J.S., Fritson, K.J., Buchel, C., Frith, C.D., Young, A.W., Calder, A.J., Dolan, R.J. (1998). A neuromodulatory role for the human amygdala in processing emotional facial expressions. *Brain, 121*, 47-57.

Morris, R.L., Erickson, C.J. (1971). Pair bond maintenance in the ring dove (*Streptopelia risoria*). *Animal Behaviour, 19*(2), 398-406.

Muller, J., Corodimas, K.P., Fridel, Z., LeDoux, J.E. (1997). Functional inactivation of the lateral and basal nuclei of the amygdala by muscimol infusion prevents fear conditioning to an explicit conditioned stimulus and to contextual stimuli. *Behavioral Neuroscience, 111*(4), 683-691.

Murray, E. A. (2007). The amygdala, reward and emotion. *Trends in Cognitive Sciences, 11*(11), 489-497.

Newman, S. W. (1999). The medial extended amygdala in male reproductive behavior: A node in the mammalian social behavior network. *Annals of the New York Academy of Sciences, 877*, 242-257.

Niels, B., Grodd, W., Diedrich, O., Klose, U., Erb, M., Lotze, M.,Schneider, F., Weiss, U., Flor, H. (1998). fMRI reveals amygdala activation to human faces in social phobias. *Neuroreport, 9*(6), 1223-1226.

Nottebohm, F., Nottebohm, M.E. (1971). Vocalizations and breeding behaviour of surgically deafened ring doves (*Streptopelia risoria*). *Animal Behaviour, 19*(2), 313-327.

Nottobohm. (2002). Neuronal replacement in adult brain. *Brain Research Bulletin, 57*(6), 737-749.

Opie, C., Atkinson, Q.D., Dunbar, R., Shultz, S. (2013). Male infanticide leads to social monogamy in primates. *Proc Natl Acad Sci, 110*(33), 13328–13332.

Ottinger, M.A., Wolfgang, M., Schleidt, E.R. (1982). Daily patterns of courtship and mating behaviors in the male Japanese quail. *Behavioural Processes, 7*(3), 223-233.

Parfitt, D.B., Newman, S.W. (1998). Fos-immunoreactivity within the extended amygdala is correlated with the onset of sexual satiety. *Hormones and Behavior, 34*(1), 17-29.

Parker, G. A. (1974). Courtship persistence and female-guarding as male time investment strategies. *Behaviour, 48*(1-2), 157-184.

Parkinson, J.A., Robbins, T.W., Everitt, B.J. (2000). Dissociable roles of the central and basolateral amygdala in appetitive emotional learning. *European Journal of Neuroscience, 12*(1), 405-413.

Patzke, N., Manns, M., Gunturkun, O. (2011). Telencephalic organization of the olfactory system in homing pigeons (*Columba livia*). *Neuroscience, 194*, 53-61.

Petrie, M., Kempenaers, B. (1998). Extra-pair paternity in birds: Explaining variation between species and populations. *Trends in Ecology & Evolution, 13*(2), 52-58.

Petrulis, A. (2009). Neural mechanisms of individual and sexual recognition in Syrian hamsters (Mesocricetus auratus). *Behavioral Brain Research, 200* (2), 260-267.

Petrulis, A., Johnston, R. E. (1999). Lesions centered on the medial amygdala impair scent-marking and sex odor recognition but spare discrimination of individual odors in female golden hamsters (Mesocricetus auratus). *Behavioral Neuroscience, 113*, 345-357.

Phan, K.L., Fitzgerald, D.A., Nathan, P.J., Tancer, M.E. (2006). Association between amygdala hyperactivity to harsh faces and severity of social anxiety in generalized social phobia. *Biological Psychiatry, 59*, 424-429.

Phelps, E.A., LeDoux, J.E. (2005). Contributions of the amygdala to emotion processing: From animal models to human behavior. *Neuron, 48*(2), 175-187.

Pitkanen, A., Savander, V., LeDoux, J.E. (1997). Organization of intra-amygdaloid circuitries in the rat: An emerging framework for understanding functions of the amygdala. *Trends in Neurosciences, 20*(11), 517-523.

Quinlan, R., Quinlan, M. (2007). Evolutionary ecology of human pair-bonds: Cross-cultural tests of alternative hypotheses. *Cross-Cultural Research, 41*(2), 149-169.

Reichard, U., Boesch, C. (Ed.). (2003). *Monogamy: Mating strategies and partnerships in birds, humans and other mammals*. Cambridge, UK: Cambridge University Press.

Reiner, A., Karten, H.J. (1985). Comparison of olfactory bulb projections in pigeons and turtles. *Brain, Behavior and Evolution, 27*(1), 11-27.

Reiner, A., Perkel, D.J., Bruce, L.L., Butler, A.B., Csillag, A., Kuenzel, W., Medina, L., Paxinos, G., Shimizu, T., Striedter, G., Wild, M., Ball, G.F., Durand, S., Guturkun, O., Lee, D.W., Mello, C.V., Powers, A., White, S.A., Hough, G., Kubikova, L., Smulders, T.V., Wada, K., Dugas-Ford, J., Husband, S., Yamamoto, K., Yu, J., Siang, C., Jarvis, E.D. (2004). Revised nomenclature for avian telencephalon and some related brainstem nuclei. *Journal of Comparative Neurology, 473*(3), 377-414.

Richardson, M.P., Strange, B.A., Dolan, R.J. (2004). Encoding of emotional memories depends on amygdala and hippocampus and their interactions. *Nature Neuroscience, 7*(3), 278-285.

Riddle, O., Dykshorn, S.W. (1932). Secretions of crop-milk in the castrate male pigeon. *Exp Biol Med (Maywood), 29*, 1213-1215.

Rissman, E. F. (1983). Detection of cuckoldry in ring doves. *Animal Behaviour, 31*(2), 449-456.

Rothe, H. (1975). Some aspects of sexuality and reproduction in groups of captive marmosets (*Callithrix jacchus*). *Zeitschrift Für Tierpsychologie (Z Tierpsychol), 37*(3), 255-273.

Sah, P., Faber, E.S.L., Lopez De Armentia, L., Power, J. (2003). The amygdaloid complex: Anatomy and physiology. *Physiological Reviews, 83*(3), 803-834.

Scheele, D., Striepens, N., Gunturkun, O., Deutschlander, S., Maier, W., Kendrick, K.M., Hurlemann, R. (2012). Oxytocin modulates social distance between males and females. *Journal of Neuroscience, 32*(46), 16074-16079.

Schwagmeyer, P.L., Mock, D. (2003). How consistently are good parents good parents? Repeatablility of parental care in house sparrow, *Passer domesticus*. *Ethology, 109*(4), 303-313.

Sharp, P. J. (1996). Strategies in avian breeding cycles. *Animal Reproduction Science, 42*, 505-513.

Shors, T.J., Townsend, D.A., Zhao, M., Kozorovitskiy, Y., Gould, E. (2002). Neurogenesis may relate to some but not all types of hippocampal-dependent learning. *Hippocampus, 12*(5), 578-584.

Shultz, R.T. (2005). Developmental deficits in social perception in autism: the role of the amygdala and fusiform face area. *International Journal of Developmental Neuroscience, 23*, 125-141.

Shultz, S., Opie, C., Atkinson, Q.D. (2011). Stepwise evolution of stable sociality in primates. *Nature, 479*, 219-222.

Silcox, A.P., Evans, S.M. (1982). Factors affecting the formation and maintenance of pair bonds in the zebra finch, *Taeniopygia guttata*. *Animal Behaviour, 30*(4), 1237-1243.

Silver, R. (1978). The parental behavior of ring doves. *American Scientist, 66*(2), 209-215.

Silver, R., Barbiere, C. (1977). Display of courtship and incubation behavior during the reproductive cycle of the male ring dove (*Streptopelia risoria*). *Hormones and Behavior, 8,* 8-21.

Silver, R., Buntin, J. (1973). Role of adrenal hormones in incubation behavior of male ring doves
(Streptopelia risoria). *Journal of Comparative and Physiological Psychology, 84*(3), 453-463.

Silver, R., Feder, H.H., Lehrman, D.S. (1973). Situational and hormonal determinants of courtship, aggressive and incubation behavior in male ring doves (*Streptopelia risoria*). *Hormones and Behavior, 4*(1-2), 163-172.

Stranahan, A.M., Khalil, D., Gould, E. (2006). Social isolation delays the positive effects of running on adult neurogenesis. *Nature Neuroscience, 9*(4), 526-533.

Stein, M.B., Goldin, P.R., Sareen, J., Eyler Zorrilla, L.T., Brown, G.G. (2002). Increased amygdala activation to angry and contemptuous faces in generalized social phobia. *Archives of General Psychiatry, 59,* 1027-1034.

Svec, L., Licht, K., & Wade, J. (2009). Pair bonding in the female zebra finch: A potential role for the nucleus taeniae. *Neuroscience, 160*(2), 275-283.

Swanson, L.W., Petrovich, G.D. (1998). What is the amygdala? *Trends in Neurosciences, 21*(8), 323-331.

Tamietto, M., & de Gelder, B. (2010). Neural bases of the non-conscious perception of emotional signals. *Nature Reviews Neuroscience, 11*, 697-709.

Thompson, R.R., Goodson, J.L., Ruscio, M.G., Adkins-Regan, E. (1998). Role of the archistriatal nucleus taeniae in the sexual behavior of male Japanese quail (*Coturnix japonica*): A comparison of function with the medial nucleus of the amygdala in mammals. *Brain, Behavior and Evolution, 51*(4), 215-229.

Tlemçani, O., Ball, G.F., D'Hondt, E., Vandesande, F., Sharp, P.J., Balthazart, J. (2000). Fos induction in the Japanese quail brain after expression of appetitive and consummatory aspects of male sexual behavior. *Brain Research Bulletin, 52*(4), 249-262.

van Praag, H., Kempermann, G., Gage, F.H. (1999). Running increases cell proliferation and neurogenesis in the adult mouse dentate gyrus. *Nature Neuroscience, 2*(3), 266-270.

Wagner, R. H. (1993). The pursuit of extra-pair copulations by female birds: A new hypothesis of colony formation. *Journal of Theoretical Biology, 163*(3), 333-346.

Wallace, C. (1908). The voices of pigeons regarded as a means of social control. *American Journal of Sociology, 14*(1), 86-100.

Walum, H., Westberg, L., Henningsson, S., Neiderhiser, J.M., Reiss, D., Igl, W., Ganiban, J.M., Spotts, E.L., Pedersen, N.L., Eriksson, E., Lichtenstein, P. (2008). Genetic

variation in the vasopressin receptor 1a gene (*AVPR1A*) associates with pair-bonding behavior in humans. *Pnas, 105*(37), 14153-14156.

Wang, Z., Yu, G., Cascio, C., Liu, Y., Gingrich, B., Insel, T.R. (1999). Dopamine D2 receptor-mediated regulation of partner preferences in female prairie voles (*Microtus ochrogaster*): A mechanism for pair bonding? *Behavioral Neuroscience, 113*(3), 602-611.

Westneat, D.F., Sherman, P.W. (1997). Density and extra-pair fertilizations in birds: A comparative analysis. *Behavioral Ecology and Sociobiology*, *41*(4), 205-215.

Wetton, J.H., Burke,T., Parkin, D.T.,Cairns,E. (1995). Single-locus DNA fingerprinting reveals that male reproductive success increases with age through extra-pair paternity in the house sparrow (*Passer domesticus*). *Proceedings of the Royal Society of London, 260*, 91-98.

Wickler, W. (1976). The ethological analysis of attachment: Sociometric, motivational and sociophysiological aspects. *Zeitschrift Für Tierpsychologie (Z Tierpsychol), 42*(1), 12-28.

Williams, J.R., Catania, K.C., Carter, C.S. (1992). Development of partner preferences in female prairie voles (*Microtus ochrogaster*): The role of social and sexual experience. *Hormones and Behavior, 26*(3), 339-349.

Winslow, J.T., Hastings, N., Cater, C.S., Harbaugh, C.R., Insel, T.R. (1993). A role for central vasopressin in pair bonding in monogamous prairie voles. *Nature, 365*, 545-548.

Wittenberger, J.F., Tilson, R.L. (1980). The evolution of monogamy: Hypothesis and evidence. *Annual Review of Ecology and Systematics, 11*, 197-232.

Wood, R.I., Newman, S.W. (1995). The medial amygdaloid nucleus and medial preoptic area mediate steroidal control of sexual behavior in the male syrian hamster. *Hormones and Behavior, 29*(3), 338-353.

Wortis, R. P. (1969). The transition from dependent to independent feeding in the young ring dove. *Animal Behaviour Monographs, 2*(1), 1-54.

Young, K. A., Gobrogge, K. L., Liu, Y, Wang, Z. (2011). The neurobiology of par bonding: Insights from a socially monogamous rodent. *Frontiners in Neuroendocrinology, 32*(1), 53-69.

Young, L., & Wang, Z. (2004). The neurobiology of pair bonding. *Nature Neuroscience, 7*, 1048-1054.

Young, L. J. (2003). The neural basis of pair bonding in a monogamous species: A model for understanding the biological basis of human behavior. *Offspring: Human Fertility Behavior in Biodemographic Perspective*, 91-397.

Young, L.J., Wang, Z., Insel, T.R. (1998). Neuroendocrine bases of monogamy. *Trends in Neurosciences, 21*(2), 71-75.

Zenone, P, Sims, M.E., Erickson, C.J. (1979). Male ring dove behavior and the defense of genetic paternity. *American Naturalist, 114*(5), 615-626.